REMARKS

I. Status of Claims

Claims 1-79 were filed with the application, and claims 2, 3, 34, 35, 38, 39, 42, 43, 46, 47, 50, 51, 54, 55 and 67-79 have been canceled. Claims 1, 4-27, 36, 37, 40, 41, 44, 45, 48, 49, 52, 53 and 56-66 are under examination, and claims 28-32 stand withdrawn. Claims 57-66 are allowed, and the remaining claims are rejected under 35 U.S.C. §112, first paragraph. The specific grounds for rejection, and applicants' response thereto, are set out in detail below.

II. Rejection Under 35 U.S.C. §112, First Paragraph

Claims 1, 4-27, 36, 37, 40, 41, 44, 45, 48, 49, 52, 53 and 56-66 stand rejected as allegedly lacking an enabling disclosure. The issues raised by the examiner are whether, in view of data that only relate to the treatment of leukemia cells using certain drug combinations, applicants' claims should be permitted to extend to solid tumors and to potentiate chemotherapeutics generally. As explained below, applicants believe that the present claims are indeed enablement and that the rejection is improper at least in light of considerable evidence arguing against the examiner's position on both of these points.

A. Tumor Cell Types Other than Leukemias

As a first response to the rejection, applicants direct the examiner to a significant body of literature on the use of CDDO and related compounds in the treatment of tumors other than leukemias, including breast, colon, pancreatic and ovarian cancers, thyroid carcinoma and metastatic melanoma:

1. Konopleva M, Lapillonne H, Clay CE, McQueen T, Studeny M, Madden T, Sporn M, Andreeff M: Activation of nuclear transcription factor PPARγ by the novel triterpenoid CDDO as targeted therapy in breast cancer. Keystone Symposium, #539, 2002.
2. Lapillonne H, Konopleva M, Tsao T, Gold D, McQueen T, Sutherland RL, Madden T, Andreeff M: Activation of peroxisome proliferator-activated receptor-γ by a novel synthetic triterpenoid 2-Cyano-3,12-dioxooleana-1,9-dien-28-oic acid induces growth arrest and apoptosis in breast cancer cells. Cancer Res 63:5926-5939, 2003.
3. Konopleva M, Lapillonne H, Shi Y-X, McQueen T, Tsao T, Gold D, Johansen M-J, Madden T, Andreeff M: Synthetic triterpenoid CDDO as a novel therapy for resistant breast cancer. Proc. AACR, Vol. 44, March 2003.
4. Zhang W, Shi Y-X, McQueen T, Hung M-C, Madden T, Sporn M, Andreeff M, Konopleva M: Synthetic triterpenoid CDDO as effective therapy for HER2-expressing resistant breast cancer. Proc. AACR, #3799, 2004.
5. Melichar B, Konopleva M, Hu W, Melicharova K, Andreeff M, Freedman RS: Growth-inhibitory effect of a novel synthetic triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, on ovarian carcinoma cell lines not dependent on peroxisome proliferator-activated receptor-γ expression. Gynecologic Oncol 93:149-154, 2004.
6. Chintharlapalli S, Papineni S, Konopleva M, Andreeff M, Samudio I, Safe S. 2-cyano-3, 12-dioxoolean-1,9-dien-28-oic acid (CDDO) and related compounds inhibit growth of colon cancer cells through peroxisome proliferators-activated receptor γ-dependent and -independent pathways. Mol Pharmacol 68:119-128, 2005.
7. Samudio I, Konopleva M, Hail Jr N, Shi Y-X, McQueen T, Hsu T, Evans R, Honda T, Gribble GW, Sporn M, Gilbert HF, Safe S, Andreeff M: 2-cyano-3,12 dioxooleana-1,9 diene-28-imidazolide (CDDO-Im) directly targets mitochondrial glutathione to induce apoptosis in pancreatic cancer. J Biol Chem 280:36273-36282, 2005.
8. Konopleva M, Zhang W, Shi Y-X, McQueen T, Tsao T, Abdelrahim M, Munsell MF, Johansen M, Yu D, Madden T, Safe SH, Hung M-C, Andreeff M. Synthetic triterpenoid CDDO induces growth arrest in HER2-overexpressing breast cancer cells. Mol Cancer Ther 5:317-328, 2006.
9. Ling X, Konopleva M, Zeng Z, Ruvolo V, Stephens LC, Schober W, McQueen T, Dietrich M, Madden TL, Andreeff M. The novel triterpenoid C-28 methyl ester of 2-cyano-3,12-dioxoolen-1, 9-dien-28-oic acid inhibits metastatic murine breast tumor growth through inactivation of signal transducers and activators of transciption 3 signaling. Cancer Res 67:4210-4218, 2007.
10. Dezube B, Kurzrock R, Eder J, Supko J, Meyer J, Camacho L, Andreeff M, Konopleva M, Lescale-Matys L, Hong D: Interim results of a phase I trail with a novel orally administered synthetic triterpenoid RTA 402 (CDDO-Me) in patients with solid tumors and lymphoid malignancies. Am. Soc. Clin. Oncol. Meeting , Vol. 25 (supp), 14101, June 20, 2007.

Because it is quite clear that these drugs work on a wide variety of cancers, ***including those forming solid tumors***, those of skill in the art would not doubt the extension of the present claims to cover cancers generally.

B. Combinations with Other Chemotherapeutics

As a second response to the rejection, applicants direct the examiner to a significant body of literature on the use of CDDO and CDDO-Me in combination with other chemotherapies s to treat leukemias, as set forth below:

11. Konopleva M, Elstner E, McQueen T, Tsao T, Estrov Z, Koeffler HP, Sporn M, Andreeff M: PPARγ nuclear receptors as a novel therapeutic target in AML. Proc. AACR, Vol. 42, March, 2001.
12. Suh W-S, Shinichi S, Kim Y, Andreeff M, Sporn M, Suh, N, Reed J: Triterpenoids CDDO and CDDO-Me down-Regulate FLIP expression and sensitize cells to TRAIL-induced apoptosis. Am. Soc. Hematol. 43rd Annual Meeting and Exposition, December, 2001.
13. Konopleva M, Lapillonne H, Lee R-M, Wang R-Y, Tsao T, McQueen T, Andreeff M: PPARγ ligand CDDO induces apoptosis in leukemias via multiple apoptosis pathways. Am. Soc. Hematol. 44th Annual Meeting, December 2002.
14. Suh W-S, Kim YS, Schimmer AD, Kitada S, Minden M, Andreeff M, Suh N, Sporn M, Reed JC: Synthetic triterpenoids activate a pathway for apoptosis in AML cells involving downregulation of FLIP and sensitization to TRAIL. Leukemia 17:2122-2129, 2003.
15. Shishodia S, Konopleva M, Andreeff M, Aggarwal BH: A synthetic triterpenoid methyl-2-cyano-3,12-dioxooleana-1,9-dien-280oate (CDDO-Me) inhibits IκBα kinase and enhances apoptosis induced by TNF and chemotherapeutic agents through downregulation of expression of NF-κB-regulated gene products in human leukemic cells. Clin Cancer Res 12:1828-1838, 2006.
16. Samudio I, Contractor R, Konopleva M, Andreeff M: The novel triterpenoid CDDOme potently synergizes with inhibition of bcl-2 function to induce apoptosis in AML via disruption of intracellular redox homeostasis, AACR, 96th Annual Meeting, April, 2005.

Because it is quite clear that various chemotherapeutics with different modes of action are potentiated by CDDO and CDDO-Me, including BH3 inhibitors, TRAIL and TRAIL receptor

antagonists, TNF, 5-FU, taxol and doxorubicin, those of skill in the art would not doubt the extension of the present claims to cover potentiation of chemotherapeutics generally.

C. Combinations Therapies to Treat Solid Tumors

Finally, applicants direct the examiner to several examples of combination therapies using CDDO-Me and a chemotherapeutic agent to treat a solid tumor cancer cell (*e.g.*, gemcitabine in pancreatic cancer; TRAIL receptor antagonists in NSCLC; doxorubicin in NSCLC, pancreatic cancer, and breast cancer):

17. Konopleva M, Samudio I, Meyer C, Ling X, Shi Y-X, McQueen T, Dietrich M, Abbruzzese J, Sporn M, Andreeff M: Pleiotropic effects of CDDO-Me mediate its single-agent antitumor effects, and its synergistc interaction with multiple chemotherapeutic modalities, EORTC [**CITE?**], 2006.
18. HGS-ETR2 in NSCLC: Declaration of Dr. Michael Andreeff (attached).
19. Doxorubicin in NSCLC, pancreatic cancer, breast cancer: Declaration of Dr. Michael Andreeff (attached).

Thus, again, it is submitted that the presently claims are fully enabled, at least in view of the enclosed evidentiary support. Reconsideration and withdrawal of the rejection is therefore respectfully requested.

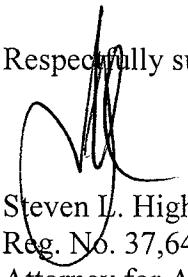
III. Rejoinder

Applicants believe that as all the claims are in condition for allowance, rejoinder of withdrawn claims 28-32, in light of the relevant linking claim(s), is appropriate at this time.

IV. Conclusion

In light of the foregoing, applicants respectfully submit that the withdrawn claims are now eligible for examination and need not be canceled. The examiner is invited to contact the undersigned attorney at (512) 536-3184 with any questions, comments or suggestions relating to the referenced patent application.

Respectfully submitted,



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Activation of nuclear transcription factor PPARγ by the novel triterpenoid CDDO as targeted therapy in breast cancer.

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The peroxisome proliferator-activated receptor (PPAR) is a nuclear receptor that is involved in the regulation of key genes of cellular homeostasis and differentiation. PPARγ must form a heterodimer with RXR to bind DNA and activate transcription. We have studied the effects of the novel PPARγ ligand CDDO (2-cyano-3,12-dioxoolen-1,9-dien-28-oic acid) in breast cancer cells. By quantitative TaqMan PCR, the breast cancer cell lines MCF-7 and MDA-MB-435 expressed PPARγ at high levels, and CDDO further increased PPARγ mRNA in MCF-7 cells. CDDO induced transactivation of PPARγ, as was evident from increase in the transcriptional activity of the luciferase reporter construct containing the PPARγ response element (PPRE). CDDO at 1μM completely inhibited growth of both, ER(+) MCF-7 and ER(-) MDA-MB-435 cells (120 hrs). Mdr-1-overexpressing MCF7/ADR cells were similarly sensitive to CDDO. CDDO induced growth inhibition through apoptosis and cell cycle arrest. In MDA-MB-231, MDA-MB-435 and MCF-7 cells CDDO induced annexin V positivity and early depolarization of the mitochondrial membrane potential, suggesting initiation of the apoptotic cascade through the mitochondrial pathway. Furthermore, CDDO downregulated mRNA expression of anti-apoptotic Bcl-2 that plays a major role in maintaining mitochondrial integrity (26-fold at 24hrs in MCF-7 cells). The RXR-specific ligand LG-100268 (100 nM) enhanced apoptosis of MDA-MB-435 cells (70% vs 46.5%). These preliminary data support the notion that simultaneous targeting of both nuclear receptors has the potential of maximizing the effects of CDDO in tumor cells. Synchronized MCF-7 and MDA-MB-435 cells showed an approximately 50% delay in entering S-phase in the presence of 1μM CDDO. While no difference was observed in p27 and cyclin E mRNA levels, marked induction of p21 mRNA (4-, 14- and 50-fold at 24, 48 and 72 hrs, respectively) was observed. Using oligonucleotide arrays, we observed over three-fold upregulation of 55 genes at 48 and 72 hrs and downregulation of 33 genes including proliferation markers cyclin D1 (5-fold), c-myc (7.8-fold), anti-apoptotic survivin (8.9-fold), and genes regulating adhesion (FGF2, FGF3, collagen IV). In a murine breast cancer xenograft model of ER(-) MDA-MB-435 cells, treatment with CDDO at 20 and 40mg/kg IV TIW induced significant reduction in tumor growth compared with the vehicle control group ($p=0.03$). In conclusion, results demonstrate that the activation of PPARγ by the novel triterpenoid CDDO provides the basis for the development of PPARγ targeted therapy of breast cancer.

2002 Keystone Symposium, Abstract 539, p. 158, 2002

[CANCER RESEARCH 63, 5926–5939, September 15, 2003]

Activation of Peroxisome Proliferator-activated Receptor γ by a Novel Synthetic Triterpenoid 2-Cyano-3,12-dioxooleana-1,9-dien-28-oic Acid Induces Growth Arrest and Apoptosis in Breast Cancer Cells[1]

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Department of Blood and Marrow Transplantation, Section of Molecular Hematology and Therapy [H. L., M. K., T. T., T. Mc., M. A.], Department of Bioinformatics [D. G.], and Department of Pharmacology [T. Ma.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030-4009, and Cancer Research Program, Garvan Institute of Medical Research, St. Vincent's Hospital, Darlinghurst, Sydney, NSW 2010 Australia [R. L. S.]

ABSTRACT

Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear hormonal receptor superfamily expressed in a large number of human cancers. Here, we demonstrate that PPARγ is expressed and transcriptionally active in breast cancer cells independent of their p53, estrogen receptor, or human epidermal growth factor receptor 2 status. 2-Cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), a novel synthetic triterpenoid, is a ligand for PPARγ. We investigated the molecular mechanisms of CDDO on proliferation and apoptosis in breast cancer cells. In all breast cancer cell lines studied, CDDO transactivated PPARγ, induced dose- and time-dependent cell growth inhibition, cell cycle arrest in G_1-S and G_2-M, and apoptosis. We then used differential cDNA array analysis to investigate the molecular changes induced by CDDO. After 16-h exposure of MCF-7 and MDA-MB-435 cells to CDDO, we found genes encoding the following proteins to be up-regulated in both cell lines: *$p21^{Waf1/CIP1}$*; *GADD153; CAAT/enhancer binding protein transcription factor family members*; and proteins involved in the ubiquitin-proteasome pathway. Among the down-regulated genes, we focused on the genes encoding *cyclin D1*, *proliferating cell nuclear antigen*, and the *insulin receptor substrate* 1. Using Western blot analysis and/or real-time PCR, we confirmed that CDDO regulated the expression of *cyclin D1*, *$p21^{Waf1/CIP1}$*, and *Bcl-2*. *Cyclin D1* and *$p21^{Waf1/CIP1}$* were additionally confirmed as important mediators of CDDO growth inhibition in genetically modified breast cancer cell lines. CDDO was able to significantly reduce the growth of MDA-MB-435 tumor cells in immunodeficient mice *in vivo*. The finding that CDDO can target genes critical for the regulation of cell cycle, apoptosis, and breast carcinogenesis suggests usage of CDDO as novel targeted therapy in breast cancer.

INTRODUCTION

Breast carcinoma is the most common malignancy among women in North America and Western Europe (1). Despite advances in earlier diagnosis and therapy, >44,000 women in the United States will die of metastatic disease each year (2). Drug resistance remains the major source of treatment failure in women with breast cancer. Two proteins implicated in drug resistance are HER2[4] and the cell cycle regulator, cyclin D1. Both are frequently overexpressed in breast cancer and are of prognostic significance (3, 4). PPARγ, a member of the nuclear hormone receptor superfamily that includes receptors for steroids, thyroid hormone, vitamin D, and retinoic acid, acts as ligand-sensitive transcription factor (5) and regulates gene transcription by binding as a heterodimer with retinoid X receptors to specific response elements (PPREs) in the promoter regions of target genes (6). Endogenous PPARγ ligands include fatty acid-like compounds such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J2, and linoleic acid (7–9). Pharmaceutical PPARγ ligands include the thiazolidinediones, which are antidiabetic drugs that include troglitazone, BRL49653 (rosiglitazone), and pioglitazone. PPARγ was first found at high levels in adipose tissue, where it functions as a critical regulator of adipocyte differentiation and fat metabolism (10–12). However, PPARγ also exists in many other cell types, where it mediates anti-inflammatory effects, modulates insulin sensitivity and inhibits cellular proliferation (13, 14). It is expressed in a large number of human cancers, including breast, colon, stomach, prostate, pancreas, bladder, placenta, lung, chondrosarcoma, and in leukemias. *In vitro* studies have demonstrated that PPARγ ligands inhibit growth and induce differentiation and apoptosis in cancer cells (7, 15–23). *In vivo* immunodeficient mice with human tumors treated with troglitazone, a pharmaceutical ligand used as an antidiabetic agent, showed similar results (18, 21, 24). In addition, in clinical trials, PPARγ ligands induced cytological and biochemical differentiation in patients with advanced liposarcoma (25) and stabilized prostate-specific antigen levels in patients with advanced prostate cancer (26).

Breast tissue, in particular, was found to express PPARγ (7, 27) in amounts greater than those found in normal breast epithelium (24). In addition, mammary tumors developed much faster in offspring of transgenic mice expressing a constitutively active form of PPARγ in breast tissue bred to transgenic animals prone to breast cancer (28). PPARγ could therefore represent a novel therapeutic target for breast cancer. PPARγ ligands exert their antitumor effects through growth inhibition and cellular differentiation. A recent study showed that PPARγ ligands inhibit the proliferation of breast cancer cells by repressing cyclin D1 expression (29). The pivotal role of cyclin D1 in the development of mammary carcinomas in mice is underscored by several lines of evidence: (*a*) transgenic mice engineered to overexpress cyclin D1 in the mammary gland develop carcinomas after a long latency period (Ref. 4; this process is accelerated by the simultaneous overexpression of c-myc), (*b*) cyclin D1 expression was required for timely epithelial cell proliferation (30, 31), and (*c*) cyclin D1-deficient mice were resistant to mammary carcinomas induced by c-neu and v-Ha-ras but not to those induced by c-myc or Wnt-1 (32). Moreover, cyclin D1 antisense oligonucleotides inhibited neu-induced

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[2] H. L. and M. K. contributed equally to this work.

[3] To whom requests for reprints should be addressed, at Department of Blood and Marrow Transplantation, Section of Molecular Hematology and Therapy, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 448, Houston, TX 77030. Phone: (713) 792-7260; Fax: (713) 794-4747; E-mail: mandreef@mdanderson.org.

[4] The abbreviations used are: HER2, human epidermal growth factor receptor 2; PPARγ, peroxisome proliferator-activated receptor γ; PPRE, PPARγ response element; CDDO, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid ; BrdUrd, bromodeoxyuridine; PI, propidium iodide; $\Delta\psi_m$, mitochondrial membrane potential; HRP, horseradish peroxidase; CMXRos, cationic lipophilic dye chlorophenyl-X-rosamine; BMG, β_2-microglobulin; FC, fold change; ER, estrogen receptor; RLU, relative light unit; PCNA, proliferating cellular nuclear antigen; C/EBP, CAAT/enhancer binding protein; CDK, cyclin-dependent kinase.

transformation and abolished the growth of Neu-transformed mammary cells in immunodeficient mice (33). Of importance, HER2-inhibitory antibodies failed to change cyclin D1 levels in HER2-overexpressing cells (34, 35).

CDDO, a novel synthetic triterpenoid, is a specific ligand for PPARγ (36). CDDO has potent differentiating, antiproliferative, and anti-inflammatory properties (37). Our group has shown that CDDO induced Bcl-2 down-regulation, mitochondrial depolarization, and caspase activation in myeloid leukemic cells (38). In the studies described here, we investigated the molecular effects of CDDO on proliferation, differentiation, and apoptosis of breast cancer cells.

MATERIALS AND METHODS

Reagents. CDDO was manufactured under the NIH RAID Program under good manufacturing practice conditions and kindly provided by Drs. Edward A. Sausville and Michael B. Sporn. A stock solution of 10 mM CDDO in DMSO was kept stored at −20°C. The working solution was prepared in DMSO and added directly to the culture medium. CDDO working concentrations varied from 0.05 to 10 μM. Appropriate amounts of DMSO (final concentration, <0.05%) were included as controls. Actinomycin D (1 μg/ml; Sigma Chemical Co., St. Louis, MO) was added to the culture 1 h before CDDO or vehicle was added.

Cell Lines and Media. MCF-7 cells were cultured in RPMI supplemented with 10% FCS (Life Technologies, Inc., Gaithersburg, MD) and L-glutamine. MDA-MB-435 and MDA-MB-231 cells were cultured in MEM with Earle's salts supplemented with 5% FCS, sodium pyruvate, nonessential amino acid solution for MEM, vitamin solution for MEM (Life Technologies, Inc.), and L-glutamine. HCT116 $p21^{Waf1/CIP1}$ and HCT116 $p21^{Waf1/CIP1}$ −/− were kindly provided by Dr. Bert Volgelstein (Johns Hopkins University, Baltimore, MD; Ref. 39) and were maintained in McCoy's 5A medium supplemented with 10% FCS and L-glutamine. T-47D breast cancer cells with constitutive cyclin D1 (D1 17-1) and a matched vector (empty) were cultured in RPMI 1640 supplemented with 5% FCS, human insulin, and gentamicin (40, 41). The cells were cultured at a density of 0.1×10^6 cells/ml in the presence or absence of indicated concentrations of CDDO. After 16–72 h, viable cells were counted using a hematocytometer and the trypan blue dye exclusion method.

Flow Cytometric Analysis of Cell Cycle. The technique of Dolbeare *et al.* (42) was used to analyze cell cycle distribution. Briefly, cells were labeled for 1 h with 50 μM BrdUrd, fixed in 70% ethanol, and kept at 4°C until processed for the detection of BrdUrd. Cells were then rehydrated in PBS, treated with 2 N HCl for 20 min, and washed extensively in blocking buffer [0.5% BSA and 0.5% Tween 20 in PBS], incubated with FITC-conjugated antibody against BrdUrd (Becton Dickinson, San Jose, CA) and diluted 1:10 in the blocking buffer for 1 h. After three washes, cells were incubated for 20 min with 1 mg/ml RNase and 1.25 μg/ml PI in 4 mM citrate buffer (pH 7.8). Cell preparations were analyzed with a FACSCalibur flow cytometer (Becton Dickinson) equipped with a 15-mW, 488-nm air-cooled argon-ion laser. The following filters were used: 530 nm (FITC) and 585 nm (PI). Data acquisition and analysis were performed using CellQuestPro software (Becton Dickinson). PCNA expression was determined using an antibody from Becton Dickinson/PharMingen.

Annexin V Staining. Cells were washed in PBS and resuspended in 100 μl of binding buffer containing Annexin V (Roche Diagnostic Corp., Indianapolis, IN). They were then analyzed by flow cytometry after the addition of PI (43). Annexin V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, and PI stains the cellular DNA of those cells with a compromised cell membrane. This allows live cells (unstained with either fluorochrome) to be distinguished from apoptotic cells (stained only with Annexin V) and necrotic cells (stained with both Annexin V and PI; Ref. 44).

Cytofluorometric Analysis of $\Delta\psi_m$. To measure $\Delta\psi_m$, cells were loaded with CMXRos (300 nM) and MitoTracker Green (100 μM; both from Molecular Probes, Eugene, OR), and the reaction was allowed to continue for 1 h at 37°C. $\Delta\psi_m$ was then determined by measuring CMXRos retention (red fluorescence) while simultaneously adjusting for the mitochondrial mass (green fluorescence; Ref. 45).

Western Blotting and Antibodies. An equal amount of cell lysate was separated by 10–12% SDS-PAGE, followed by immunoblotting on Hybond-P membranes (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). Proteins were visualized using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) after incubation for 2 h or overnight with the following primary antibodies: human cyclin D1 (mouse monoclonal HD-11), human cyclin E (rabbit polyclonal M-20), human cdk4 (rabbit polyclonal C-22), human cdk2 (mouse monoclonal D-12), human $P21^{Waf1/CIP1}$ (mouse monoclonal 187), and PPARγ (mouse monoclonal E-8) from Santa Cruz Biotechnology (Santa Cruz, CA); human p27 (mouse monoclonal 554069) and human pRb (mouse monoclonal G3-245) were from PharMingen, San Diego, CA.

Quantitative Real-Time Reverse Transcription-PCR. Total RNAs were prepared using Trizol reagent as described by the manufacturer (Life Technologies, Inc.). One μg of total RNA was reverse transcribed by avian myeloblastosis virus reverse transcriptase (Roche Diagnostic Corp.) under standard conditions. Duplicate samples of 1 μl of each cDNA were amplified by PCR in the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). The Primer Express program (PE Applied Biosystems) was used to design the primers and probes. The amplification reaction mixture (25 μl) contained cDNAs, forward primers, reverse primers, probes, and Taqman Universal PCR Master Mix (PE Applied Biosystems). BMG was coamplified as an internal control to normalize for variable amounts of cDNA in each sample. The thermocycler parameters were as follows: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s; and 60°C for 1 min. Results were collected and analyzed to determine the PCR cycle number that generated the first fluorescence signal above a threshold (threshold cycle, C_T; 10 SDs above the mean fluorescence generated during the baseline cycles), after which a comparative C_T method was used to measure relative gene expression. The following formula was used to calculate the relative amount of the transcript of interest in the treated sample (X) and the control sample (Y), both of which were normalized to an endogenous reference value (BMG): $2^{-}\Delta\Delta CT$, where ΔC_T is the difference in C_T between the gene of interest and BMG, with the $\Delta\Delta C_T$ for sample $X = \Delta C_T(X) - \Delta C_T(Y)$. The oligonucleotide and probe sequences used are listed in Table 1.

Plasmids. For the luciferase assays, response elements were cloned into a TK-LUC reporter that contains the herpes virus thymidine kinase promoter. The response element consensus sequence for PPRE was kindly provided by Dr. Ronald M. Evans (9). Full-length human PPARγ1 (kindly provided by Dr. Krishna K. Chatterjee; Ref. 46) was cloned into the pEGFP-C2 vector (Clontech).

Transfection Assays. One day before transfection, cells were plated at a density of 0.1×10^5 to 2×10^5 cells/ml. Cells were transfected with PPREx3-TK-LUC reporter (300 ng/10^5 cells) or with 1 μg of pEGFP-C2 or pEGFP-C2hPPARγ using the Fugene-6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. For the luciferase

Table 1 *Oligonucleotide and probe sequences used for quantitative real-time PCR*

Oligonucleotide sequence (5′–3′)	
Cyclin D1 FP	TCCATCCGGCCCGAG
Cyclin D1 RP	GAGCTTGTTCACCAGGAGCAG
Cyclin D2 FP	CTACATGCGCAGAATGGTGG
Cyclin D2 RP	AGCACCCAGGAGTTGCAGAT
Cyclin E FP	CCCATCCTTCTCCACCAAAG
Cyclin E RP	CCCTGTTTGATGCCATCCAC
$P21^{Waf1/CIP1}$ FP	CGCTAATGGCGGGCTG
$P21^{Waf1/CIP1}$ RP	CGGTGACAAAGTCGAAGTTCC
$P27^{KIP1}$ FP	GAGGACACGCATTTGGTGG
$P27^{KIP1}$ RP	GCTCCTCTAACCCCGTCT
PPARγ FP	GGCTTCATGACAAGGGAGTTTC
PPARγ RP	AACTCAAACTTGGGCTCCATAAAG
β2MG FP	AGCTGTGCTCGCGCTACTCT
β2MG RP	TTGACTTTCCATTCTCTGCTGG
Probe sequence 5′(FAM)-(TAMRA)3′	
Cyclin D1 probe	CTGCTGCAAATGGA
Cyclin D2 probe	TGTGAGGAACAGAAGTGCGAAGAAGAGGTC
Cyclin E probe	AGTTGCGCGCCTGCTCCACG
$P21^{Waf1/CIP1}$ probe	ATCCAGGAGGCCCGTGAGCGA
$P27^{KIP1}$ probe	CCCAAAGACTGATCCGTCGGACAGC
PPARγ probe	AAAGAGCCTGCGAAAGCCTTTTGGTG
β2MG probe	TCTTTCTGGCCTGGAGGGCATCC

FP: forward primer; RP: reverse primer.

assay, transfected cells were treated with 1 μM CDDO or with vehicle for 24 h starting on day 1 after transfection. Cells were then harvested, and the luciferase assay was performed using Fluoroscan Ascent (Labsystems, Franklin, MS). MCF-7/pEGFP-C2 and MCF-7/pEGFP-C2hPPARγ stable transfectants were selected in the presence of G418 (800 μg/ml), and individual clones were isolated by limited dilution. In lysates from the selected clones, evaluated for transgene expression by immunoblot analyses using anti-PPARγ antibody, the fusion protein was detected at M_r 80,000.

Oligonucleotide Array-based Expression Profiling. Hybridization was performed using Human Genome U95Av2 probe arrays (Affymetrix, Santa Clara, CA) containing probe sets from ~12,000 previously characterized genes.[5] The target was labeled and hybridized to the probe arrays, washed, stained, and scanned as described previously. Briefly, total RNAs were prepared using the RNeasy Total RNA Isolation kit (Qiagen, Valencia, CA) and double-stranded cDNA was synthesized from total RNA; an *in vitro* transcription reaction was done to produce biotin-labeled cRNA from the cDNA; and the cRNA was fragmented and hybridized to the oligonucleotide probes on the probe array during 16 h of incubation at 45°C. Immediately after hybridization, the hybridized probe array underwent an automated washing and staining protocol on the fluidics station, and the Genechip Microarray Suite 4.0 Software (Affymetrix) was used to measure the intensity of expression of each feature. DNA-Chip Analyzer software was designed to better analyze the quantified image (47, 48). The expression value for each target gene was determined by calculating the average of differences (perfect-match intensity minus mismatch intensity) of the 14–20 probe pairs used for the particular gene. Ratios of the average proportion of treated cells to control cells were determined in the respective experiments.

Statistical Analysis. DNA-Chip Analyzer software was used to analyze the quantified images. The expression change for each target gene was estimated with the reduced Li Wong p.m.-MM difference model (47, 48). The statistical results for each target gene and the contrasting expression levels included the FC, 90% confidence interval on the FC, the difference in means or difference in mean expression, and the P testing the hypothesis H_0: FC = 1 *versus* the alternative H_a: FC ≠ 1. The statistical results were imported into the Result Viewer 2.0 software (49). This application allows investigators to browse through the results of bioinformatics experiments in a user-friendly environment to extract records based on a statistical or biological criterion.

***In Vivo* Studies.** Female nude immunodeficient mice were purchased from Harlan Laboratory (Indianapolis, IN). Two groups of nude mice (4–6 weeks) were inoculated s.c. with MDA-MB-435 cells (n = 25; 2 × 10^6 cells/mouse in 100 μl of PBS). Ten days after inoculation, one group (n = 10) of mice was treated with 40 mg/kg CDDO (sodium salt CDDO prepared as follows: 2 mg of CDDO, 0.6 mg of sodium carbonate, 0.84 mg of sodium bicarbonate, 7 mg of sodium chloride, and sodium hydroxide/hydrochloric acid to adjust the pH to 9.6) i.v. twice a week for 3 weeks. The rest of the mice (n = 15) received vehicle only. Tumors were measured twice weekly with microcalipers, and the tumor volume was calculated as length × width.

RESULTS

PPARγ Is Expressed in Breast Cancer Cell Lines. First, we investigated the basal mRNA expression of *PPARγ* in five different breast cancer cell lines: MCF-7; SKBR-3; MDA-MB-231; MDA-MB-435; and MDA-MB-453. Quantitative real-time reverse transcription-PCR showed that *PPARγ* mRNA was expressed in all cell lines studied irrespective of their ER, HER2/neu, or p53 status. The highest *PPARγ* mRNA expression was in MDA-MB-231 cells, which are highly metastatic in mouse models. These mRNA expression data were then confirmed by Western blot analysis in MDA-MB-231, MDA-MB-435, and MCF-7 cells, with MDA-MB-231 and MDA-MB-435 cells expressing the highest levels of the receptor (Fig. 1). These three cell lines were therefore chosen for subsequent analyses. As CDDO (1 μM) increased *PPARγ* mRNA expression in both MCF-7 and MDA-MB-435 cells by 2-fold at 24 h and by >7-fold at 48 and 72 h, we then investigated whether CDDO could efficiently

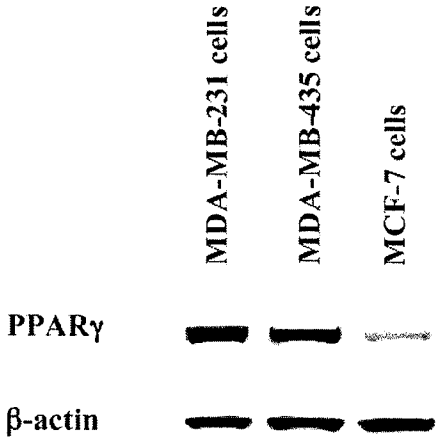

Fig. 1. PPARγ expression in human breast cancer cell lines analyzed by Western blot. Total protein extract from the indicated cell lines was analyzed using a monoclonal antibody against human PPARγ.

transactivate *PPARγ*. To determine this, cells were transfected with the PPRE-TK-LUC reporter, and luciferase assays were performed. In the absence of CDDO, the PPRE reporter was activated in all cell lines in a wide range, from low in MDA-MB-435 cells (0.17 RLU) to high in MCF-7 cells (8.71 RLU). In the presence of CDDO, however, PPRE was activated by >10-fold in all three cell lines studied (Fig. 2). These experiments thus showed the ability of CDDO to transactivate *PPARγ*.

CDDO Inhibits Growth of MCF-7, MDA-MB-435, and MDA-MB-231 Breast Cancer Cells. MCF-7, MDA-MB-435, and MDA-MB-231 cells were exposed to different concentrations of CDDO (0.1, 0.5, 1, and 10 μM) for 72 h. The effects of CDDO on the growth of breast cancer cells were then determined by cell counts at 24, 48, and 72 h (Fig. 3*A*). Untreated ER(−) MDA-MB-231 and MDA-MB-435 cells proliferated much faster than ER(+) MCF-7 cells, as shown by comparison with the growth curve of these cells in the presence of vehicle only. At 0.1 μM, CDDO only modestly retarded cell growth. At 0.5 and 1 μM, CDDO significantly inhibited cell growth 8-, 7-, and 4-fold in MDA-MB-231, MDA-MB-435, and MCF-7 cells, respectively, at 72 h; the cells became larger and remained predominantly adherent. At 10 μM CDDO, cell growth inhibition was more pronounced but cells were mostly (at 24 and 48 h) to totally (at 72 h and all subsequent time points) detached and became smaller and round. For the experiments described in the following sections, a fully cytostatic concentration of CDDO of 1 μM was used.

To determine the correlation between PPARγ expression and the sensitivity of breast cancer cells to CDDO, we tested the effects of CDDO in PPARγ-overexpressing MCF-7 cells. Vector control or EGFP-PPARγ-transfected MCF-7 cells were treated with 0.05, 0.1, and 0.5 μM CDDO for 24, 48, and 72 h. As shown in Fig. 3*B*, overexpression of PPARγ enhanced the sensitivity of cells to growth arrest induced by low concentrations of CDDO (0.05 and 0.1 μM), but at 0.5 μM cells, complete inhibition of cell growth was observed in both cell lines.

CDDO Induces Cell Cycle Arrest in MCF-7, MDA-MB-435, and MDA-MB-231 Cell Lines. Because CDDO inhibited cell growth, we investigated its effects on cell cycle distribution. The proliferation and repartition of cells in different phases of the cell cycle was analyzed at 24, 48, and 72 h on the basis of BrdUrd incorporation. The percentage of cells in S phase was defined after double staining with BrdUrd/PI and proved to be higher in MDA-MB-435 and MDA-MB-231 cells than in MCF-7 cells (60, 44, and

[5] Internet address: http://www.netaffx.com.

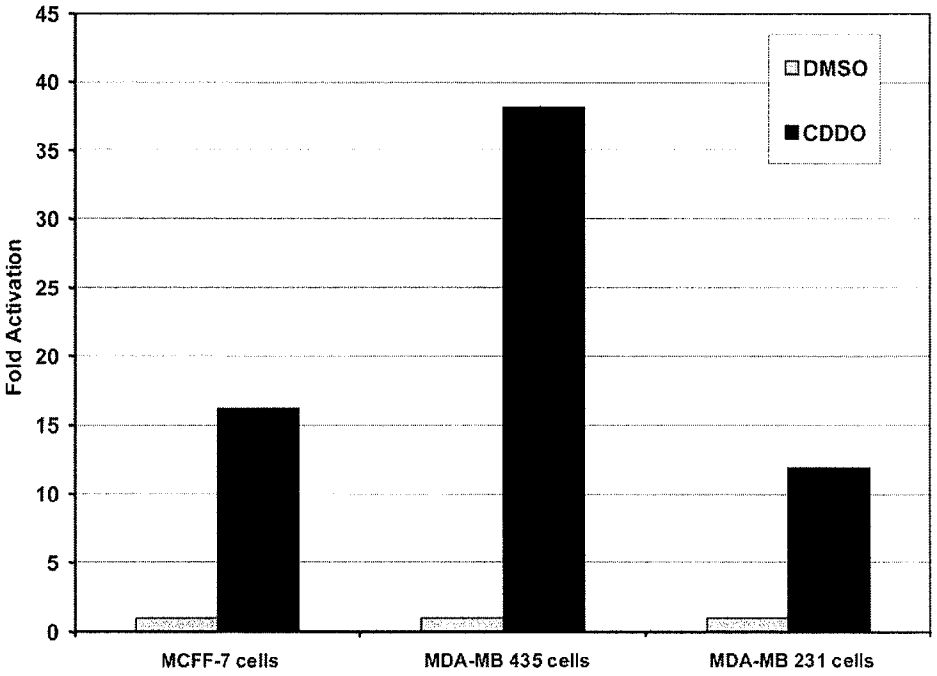

Fig. 2. Activation of the PPRE by CDDO. CDDO activates the PPRE reporter in MDA-MB-231, MDA-MB-435, and MCF-7 cell lines. The cells were transfected with the PPRE x3-TK-LUC reporter (300 ng/10^5 cells) for 24 h followed by 24 h treatment with CDDO (2 μM) or vehicle. Normalized luciferase activity was determined and is shown as the fold activation relative to results in vehicle-treated cells.

39%, respectively), which is in concordance with the observed enhanced cell growth. Although DMSO did not affect proliferation, 1 μM CDDO progressively reduced proliferation in all three cell lines tested. Specifically, BrdUrd incorporation and the percentage of cells in S phase decreased dramatically and reached complete G_1-S and G_2-M blocks at 48 h in MCF-7 and MDA-MB-231 cells and at 72 h in MDA-MB-435 cells. All results are summarized in Fig. 4. These data demonstrated that CDDO rapidly inhibits BrdUrd incorporation at the G_1-S transition and dramatically reduced the proportion of cells in S phase in a time-dependent manner. In addition, cells accumulated in G_2-M.

CDDO Induces Apoptosis at Concentrations >1 μM. As CDDO has been shown to induce apoptosis in leukemia cell lines and primary leukemia samples (38), we studied the effects of CDDO on apoptosis induction in breast cancer cell lines. As determined by Annexin V staining and flow cytometry, 1 μM CDDO induced apoptosis in MDA-MB-231 cells (DMSO = 2%, CDDO = 18% at 48 h and 41% at 72 h), MDA-MB-435 cells (DMSO = 5%, CDDO = 19% at 72 h), and MCF-7 cells (DMSO = 8%, CDDO = 29% at 24 h). At lower CDDO concentrations of 0.1 and 0.5 μM, apoptosis was not induced. At 2 μM CDDO, apoptosis was induced after the same pattern seen for 1 μM.

To determine the molecular changes that occur during CDDO-induced cell death, we first studied $\Delta\psi_m$. Exposure to 1 μM CDDO induced loss of $\Delta\psi_m$ in all three cell lines studied: MDA-MB-231 cells (DMSO = 5%, CDDO = 17% at 48 h and 48% at 72 h); MDA-MB-435 cells (DMSO = 8%, CDDO = 24% at 48 h and 58% at 72 h); and MCF-7 cells (DMSO = 8%, CDDO = 18% at 24 h, 28% at 48 h, and 34% at 72 h). These changes were followed by translocation of phosphatidylserine in all three cell lines. At 10 μM CDDO, the $\Delta\psi_m$ was lost in all three cell lines at 72 h. Fig. 5 shows a representative experiment. These data demonstrate that CDDO induces apoptosis mediated through the mitochondrial pathway in breast cancer cell lines.

Genes Targeted by CDDO. We then studied the effects of CDDO on a larger number of genes using an Affymetrix oligo array. MCF-7 and MDA-MB-435 cells were treated with 2 μM CDDO or with vehicle alone as control for 16 h. RNA was isolated, labeled, and hybridized to the oligonucleotide microarrays containing probe sets representing ~12,000 human genes. The following criteria were used to select the genes differentially expressed in treated and control cells: lower bound of FC > 2 and difference of means >150 for up-regulated genes and upper bound of FC < −2 and difference of mean <− 150 for down-regulated genes. For the MCF-7 cells, 280 genes showed a >2-fold increase and 188 genes showed a ≤2-fold decrease. For the MDA-MB-435 cells, 208 genes were up-regulated >2-fold, and 293 genes were down-regulated ≤2-fold. To investigate the molecular changes induced by CDDO, we first selected the genes that were up-regulated or down-regulated in both cell lines. Fifty genes were up-regulated >2-fold, and 41 genes were down-regulated ≤2-fold in both MDA-MB-435 and MCF-7 cell lines. These genes were then classified by function and are summarized in Tables 2 and 3. We specifically focused on genes involved in cell cycle regulation and apoptosis and found that CDDO down-regulated the genes encoding *cyclin D1*, *PCNA*, and *cyclin F* and up-regulated the genes encoding $p21^{Waf1/CIP1}$ and *CDC-like kinase 1*. CDDO up-regulated the apoptotic gene *GADD153* (TLS/cyclophosphamide-Adriamycin-vincristine-prednisone) and down-regulated the *IEX-1* gene in both cell lines. Some of the cell cycle and apoptotic genes were differentially expressed in only one cell line such as the genes encoding *GADD34* and *GADD45*, which were up-regulated, and the genes encoding *Bcl-2*, *CDC25B*, and *cyclin A2*, *B1*, and *B2*, which were down-regulated.

To confirm the microarray results, changes in mRNA expression of different genes were analyzed by real-time PCR. We tested the expression of *cyclin D1*, *cyclin D2*, *cyclin E*, $p21^{Waf1/CIP1}$, $p27^{KIP1}$, and *Bcl-2* (Table 4). In both cell lines, *cyclin D1* mRNA was down-regulated, and $p21^{Waf1/CIP1}$ mRNA was up-regulated. No significant differences in *cyclin E* and $p27^{KIP1}$ mRNA were found, and no *cyclin D2* mRNA expression was detected. *Bcl-2* mRNA expression was down-regulated 20-fold by real-time PCR only in MCF-7 cells, a finding also demonstrated by microarray analysis, which showed a 7-fold decrease. Fig. 6 shows the changes in *cyclin D1* and $p21^{Waf1/CIP1}$ mRNA expression detected by microarray and real-time

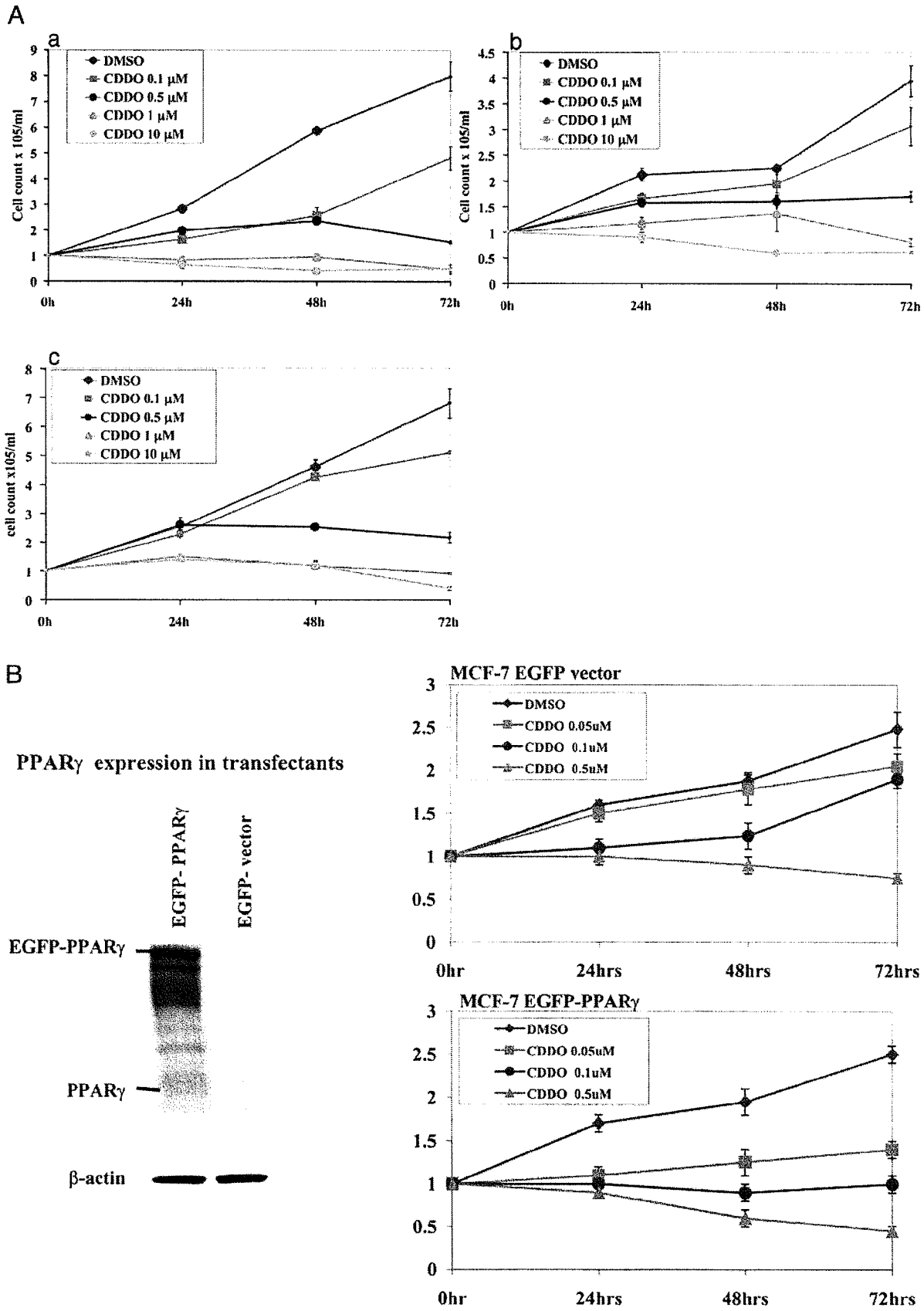

Fig. 3. *A*, inhibition of cell growth by CDDO in MDA-MB-435, MDA-MB-231, and MCF-7 breast cancer cell lines. MDA-MB-231 (*a*), MCF-7 (*b*), and MDA-MB-435 (*c*) cells were incubated with different concentrations (0.1, 0.5, 1, and 10 μM) of CDDO or DMSO for 24, 48, and 72 h. The effect on cell growth was determined by cell counts. One μM CDDO exerts a fully cytostatic effect. *B*, MCF-7 cells were transfected with pEGFP vector or pEGFP-PPARγ1. The fusion protein was detected at M_r 80,000 as shown by Western blot using anti-PPARγ antibody. The effect on cell growth was determined by cell counts. CDDO was more effective in PPARγ-overexpressing cells.

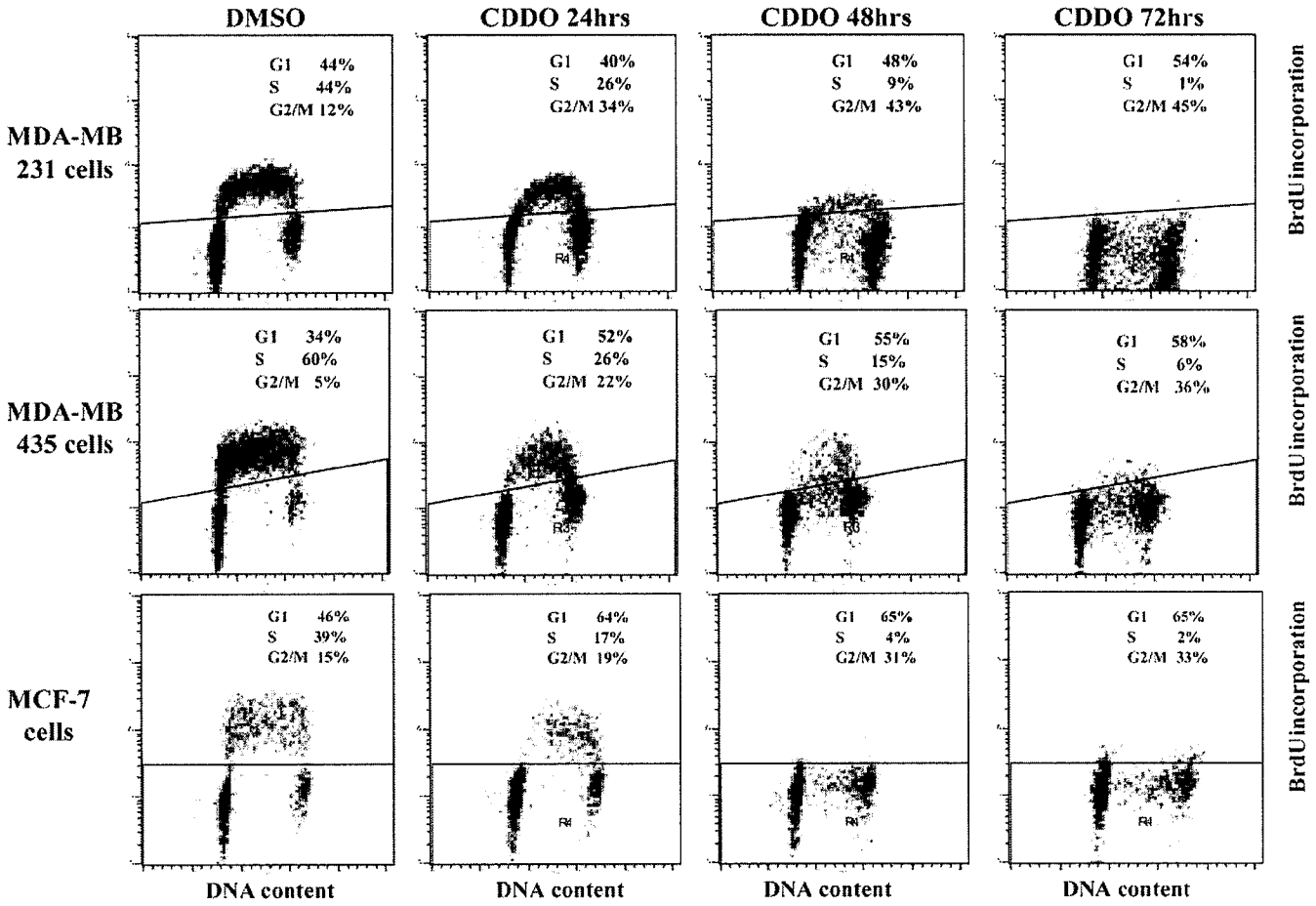

Fig. 4. CDDO induction of cell cycle arrest in MDA-MB 435, MDA-MB-231, and MCF-7 breast cancer cell lines. The percentages of cells in each phase of the cell cycle was determined by double fluorescence BrdUrd/PI fluorescence-activated cell sorting analysis of samples of 10,000 cells each. For each time point, a negative control without BrdUrd incorporation was analyzed and defined background positivity, which is represented by a line on the images. CDDO inhibited proliferation in all three cell lines, as shown by a drastic decrease of cells in S phase and an accumulation in G_2-M.

PCR. The decrease in PCNA was confirmed by flow cytometry (data not shown).

Other genes besides those involved in cell cycle and apoptosis regulation were found to be modulated by CDDO. This included genes encoding *C/EBP* transcription factors, zinc finger proteins, proteins involved in the ubiquitin-proteasome pathway, some heat shock proteins, which were up-regulated, genes encoding members of the histone family and some proteins involved in lipid metabolism and the regulation of insulin, which were down-regulated (Tables 2 and 3). Among the genes known to be regulated by PPARγ, some were differentially expressed only in MDA-MB-435 cells such as adipsin, adipophilin, clusterin, and *GLUT3*, which were up-regulated, and different insulin-like growth factors, fatty acid enzyme, and tumor necrosis factor α, which were down-regulated (50–52). This cell line appears more sensitive to CDDO with regard to lipid and glucose regulation.

CDDO Reduces Cyclin D1 Expression and Induces $p21^{Waf1/CIP1}$ Expression in Breast Cancer Cell Lines. Of the genes regulated by CDDO and identified by microarray studies, some such as those encoding *cyclin D1* and $p21^{Waf1/CIP1}$ play a critical role in breast cancer. To further elucidate our findings, we used real-time PCR to analyze the differential expression of mRNA for *cyclin D1*, *cyclin E*, $p21^{Waf1/CIP1}$, $p27^{KIP1}$, and *Bcl-2* at 24, 48, and 72 h in the presence of 1 μM CDDO or vehicle in MDA-MB-435, MDA-MB-231, and MCF-7 cells. Results are summarized in Table 5. Of note, *cyclin D1* mRNA expression was down-regulated in all three breast cancer cell lines, and this was completely prevented by pretreatment with actinomycin D. In addition, *Bcl-2* mRNA expression was reduced in MCF-7 cells at 24 and 48 h and in MDA-MB-435 cells at 72 h. In contrast, $P21^{Waf1/CIP1}$ mRNA was highly induced in all three cell lines in a time-dependent manner (Fig. 7). This up-regulation was markedly (10-fold) but not completely inhibited by pretreatment with actinomycin D (1.7-, 2.9-, and 5.3-fold higher levels of $P21^{Waf1/CIP1}$ at 24, 48, and 72 h, respectively). Western blot analysis demonstrated corresponding changes at the protein level for $P21^{Waf1/CIP1}$ and cyclin D1 in MDA-MB-435 and MCF-7 cells (Fig. 8). However, expression levels of CDK2 and CDK4 did not change, nor did cyclin E (data not shown). Of interest, $p27^{KIP1}$ was up-regulated 2-fold in the presence of CDDO, suggesting posttranscriptional regulation. Taken together, these data suggest that CDDO can efficiently target the cell cycle regulators cyclin D1 and $P21^{Waf1/CIP1}$ and partially affect $p27^{KIP1}$, all known to be clinically relevant in human breast cancer.

Cyclin D1 and $P21^{Waf1/CIP1}$ Are Critical Mediators of CDDO-induced Growth Inhibition. To determine whether cyclin D1 down-regulation is critical for CDDO-induced growth inhibition, we conducted experiments in T-47D breast cancer cells with constitutive cyclin D1 overexpression. T-47D cyclin D1 17-1 showed 5-fold overexpressed cyclin D1 at the protein level compared with matched controls (T-47D empty; Ref. 40; Fig. 9). Cells were exposed to different concentrations of CDDO (0.1, 0.5, and 1 μM) for 72 h, and

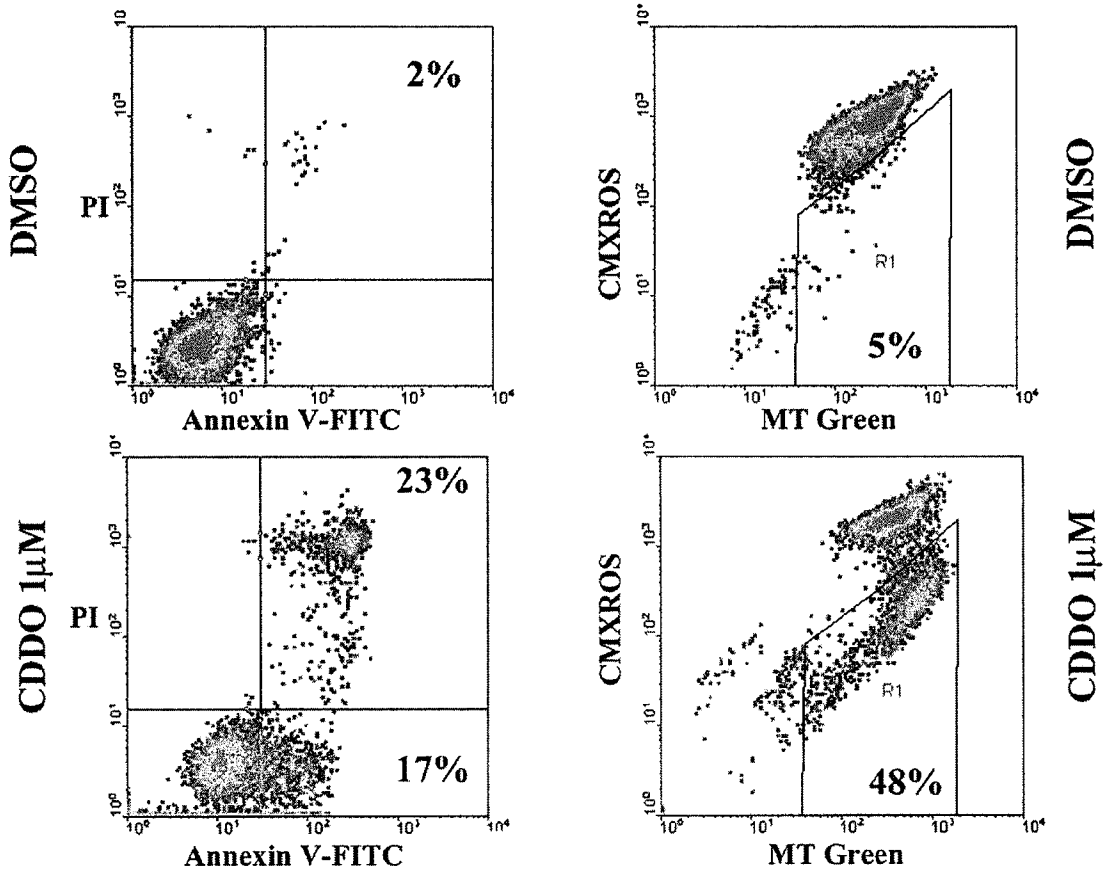

Fig. 5. Induction of apoptosis and a decrease in the $\Delta\Psi_m$ in human breast cancer cell lines by CDDO. Results are shown for MDA-MB-231 cells. Cells were grown in the presence of 1 μM CDDO or vehicle for 72 h. *A*, apoptosis was measured by staining with FITC-labeled Annexin V, which binds to phosphatidylserine with high affinity. Cells with positivity for Annexin V and excluding PI are apoptotic (*bottom right quadrant*); double positive cells have undergone secondary necrosis (*top right panel*). *B*, the CMXRos assay was performed to evaluate the $\Delta\Psi_m$ and shows CDDO-induced loss of $\Delta\Psi_m$.

cell growth inhibition and regulation of cyclin D1 were analyzed. Cyclin D1-overexpressing cells were significantly less sensitive to CDDO at 24 and 48 h, whereas the difference was minimal at 72 h (Fig. 9). Of importance, 0.5 μM CDDO at 72 h down-regulated cyclin D1 expression in both T-47D cyclin D1 17-1 and control cells (Fig. 9).

To analyze the role of $p21^{Waf1/CIP1}$ for the sensitivity to CDDO, we carried out experiments in p21-knockout HCT116 cells (39). The $p21^{Waf1/CIP1}$ parental and knockout cells were exposed to different concentrations of CDDO (0.1, 0.5, and 1 μM) for 72 h. Although p21 was undetectable in $p21^{Waf1/CIP1}$ HCT116-knockout cells, it was expressed in parental cells and further induced by CDDO (Fig. 10). At 72 h, $p21^{Waf1/CIP1}$-knockout HCT116 cells were moderately resistant to 0.5 μM CDDO compared with control cells, but this effect was lost at higher (1 μM) concentration (Fig. 10). Cell cycle analysis showed that after CDDO, 10% of cells remained in S phase in parental HTC116 cells, whereas 35% of the cells remained BrdUrd positive in $p21^{Waf1/CIP1}$-knockout cells (Fig. 10).

CDDO Treatment Reduces Breast Cancer Growth in Immunodeficient Mice. The effect of CDDO on breast tumors was further tested in female nude immunodeficient mice (n = 25), inoculated s.c. with 2 × 10^6 MDA-MB-435 cells each. Once the tumors were established 10 days after inoculation, 10 mice were treated with CDDO at a dose of 40 mg/kg i.v. twice a week for 3 weeks. The rest of the mice (n = 15) received vehicle only. The tumors were measured twice weekly with microcalipers, and the tumor volume was calculated as the length × width. The results demonstrated a statistically significant (P = 0.013) reduction in tumor growth in the treated group compared with the vehicle group (Fig. 11).

DISCUSSION

In this study, we investigated the effects of a recently characterized PPARγ ligand, CDDO, in breast cancer cell lines. PPARγ ligation is known to induce differentiation, growth arrest, and apoptosis and to inhibit angiogenesis in certain tumors. In the present study, we demonstrated that *PPARγ* was expressed at high levels in all breast cancer cell lines studied, independently of their ER, p53, and HER2/neu status. Of additional importance, *PPARγ* was transcriptionally active, and CDDO, as with other PPARγ ligands, induced *PPARγ* transactivation in the luciferase reporter assay. We then studied the effects of CDDO on breast cancer cell growth *in vitro*. CDDO at 1 μM completely abrogated tumor cell growth. This was noted in all breast cancer cell lines studied, including ER(−), p53-mutated, and HER2-expressing cells. To shed light on the mechanisms of the growth arrest produced by CDDO, we investigated its effect on cell cycle regulation and apoptosis. CDDO induced a complete G_1-S block and an accumulation of cells in G_2. Growth inhibition, cell cycle block, and apoptosis induction were all more pronounced in MDA-MB-231 cells overexpressing PPARγ, which showed rapid cell growth. Furthermore, overexpression of PPARγ in MCF-7 cells sensitized cells to low concentrations of CDDO. However, at high concentrations, complete growth arrest was observed in both cell lines, pointing out to PPARγ-independent and PPARγ-dependent mechanisms. To elucidate the molecular changes induced by CDDO, we performed differential cDNA array analysis in two different cell lines (MCF-7 and MDA-MB-435 cells). Of the >12,000 genes represented on the chips, 91 genes were changed in both cell lines. We then characterized the

Table 2 *Summary of genes down-regulated in both MDA-MB-435 and MCF-7 cells after exposure to CDDO (16 h, 2 μM)*

GenBank accession no.	FC MCF-7 cells	Gene name	FC in MDA-MB-435 cells
Cell cycle			
X59798	−7.0	Cyclin D1	−3.1
Z36714	−2.0	Cyclin F	−3.3
M15796	−3.3	Proliferating cell nuclear antigen	−2.3
Transcription			
Z97630	−5.7	H1 histone family member 0	−26.3
D64142	−4.8	H1 histone family member X	−10.2
Z83738	−4.8	H2B histone family member E	−2.6
AA255502	−2.0	H4 histone family member G	−2.3
AL037557	−2.7	Polymerase (RNA) II (DNA directed) polypeptide I	−2.3
X75755	−3.4	Splicing factor arginine/serine-rich 2	−2.1
AJ245416	−2.9	U6 snRNA-associated Sm-like protein	−2.6
X79865	−2.9	Mitochondrial ribosomal protein L12	−2.6
Apoptosis			
S81914	−5.1	Immediate early response 3, IEX-1	−3.4
Y07846	−2.0	GAS2-related on chromosome 22	−2.3
Oncogene			
V00568	−5.7	v-myc avian myelocytomatosis viral oncogene	−2.5
AB019527	−2.2	Leucine zipper down-regulated in cancer 1	−2.0
Transporter			
U81375	−5.6	Solute carrier family 29 member 1	−2.0
D38076	−2.1	RAN binding protein 1	−2.5
Transcription factor			
AL109701	−2.3	CREBBP/EP300 inhibitory protein 1	−2.2
L23959	−2.6	Transcription factor Dp-1	−2.0
AA845349	−2.6	Thyroid hormone receptor interactor 7	−3.9
Cytoskeleton/cell adhesion			
J00314	−3.0	Tubulin beta polypeptide	−4.1
X02344	−2.1	Tubulin beta 2	−2.1
M94362	−2.6	Lamin B2	−2.0
L37747	−2.2	Lamin B1	−3.4
U53204	−2.4	Plectin 1 intermediate filament binding protein 500kD	−5.3
Metabolism			
Y09008	−2.7	Uracil-DNA glycosylase	−3.1
U31930	−2.2	dUTP pyrophosphatase	−2.8
M36067	−2.1	Ligase I DNA ATP-dependent	−2.8
J04031	−3.4	Methylenetetrahydrofolate dehydrogenase	−2.4
D78586	−2.7	Carbamoyl-phosphate synthetase 2	−2.1
U84371	−2.3	Adenylate kinase 2	−2.0
AF014398	−2.8	Inositol(myo)-1(or 4)-monophosphatase 2	−4.4
M21154	−2.2	S-adenosylmethionine decarboxylase 1	−2.8
D63391	−2.1	Platelet-activating factor acetylhydrolase isoform Ib gamma	−2.9
Lipid metabolism			
AF034544	−2.0	7-dehydrocholesterol reductase	−2.5
L00352	−3.0	Human LDL receptor	−3.0
AI557240	−2.6	Diazepam binding inhibitor	−2.2
Signal transduction			
S62539	−8.2	Insulin receptor substrate 1	−7.7
M62403	−2.1	Insulin-like growth factor-binding protein 4	−3.1
Others			
D50914	−2.2	Block of proliferation 1	−2.0
X57351	−2.0	Interferon induced transmembrane protein 2 (1–8D)	−2.1

common differentially expressed genes regulating cell cycle and/or apoptosis.

cDNA array studies showed that CDDO regulated the genes encoding *cyclin D1*, *PCNA*, and $p21^{Waf1/CIP1}$ and proteins involved in the ubiquitin-proteasome pathway, changes consistent with the observed cell cycle arrest. These proteins play important roles in cell cycle regulation. Specifically, cyclin D1 participates in the control of G_1 progression by activating its kinase partners, CDK4 and CDK6, which leads to the phosphorylation of the retinoblastoma protein, thereby relieving pRb's inhibitory function (53). The overexpression of cyclin D1 accelerates the passage of cells through G_1, whereas the inhibition of cyclin D1 leads to G_1 arrest (54, 55). Cyclin D1 was shown to be rate limiting for cell cycle progression in breast epithelial cells (56). The down-regulation of cyclin D1 observed in our experiments suggests an important effect of CDDO on cell cycle control of breast cancer. A recent study demonstrated that the inhibition of proliferation by PPARγ ligands is mediated by the PPARγ-dependent repression of cyclin D1. Of interest, we demonstrated increased resistance to CDDO at early time points for cyclin D1 overexpressing breast cancer cells, but cyclin D1 was still down-regulated at 72 h. This observation is reminiscent of results obtained with antiestrogens (40). Repression of cyclin D1 involves the competition between PPARγ and c-Fos for limited quantities of p300, a coactivator protein also known as CBP (29). Furthermore, because many cell cycle regulators are controlled by ubiquitin-proteasome degradation, the down-regulation of the cyclin D1 protein by CDDO could be mediated by this pathway in addition to transcriptional down-regulation because we found the induction of mRNAs for some of the proteins involved in this pathway (57). This hypothesis was recently confirmed in MCF-7 cells treated by PPARγ agonists ciglitazone or 15-deoxy-Δ12,14-prostaglandin J2 (58). Targeting cyclin D1 may be of critical importance considering its pivotal role in human breast cancers. Cyclin D1 protein is overexpressed in 50% of human mammary carcinomas (59–61). Importantly, transgenic mice engineered to overexpress cyclin D1 in the mammary gland develop carcinomas after a long latency period, a process that is accelerated by simultaneous overexpression of c-myc (40, 41), and cyclin D1-deficient mice are resistant to mammary carcinomas induced by c-neu and v-Ha-ras (but not to cancers induced by c-myc or Wnt-1; Ref. 32). We report here that CDDO down-regulates cyclin D1 in all three cell lines studied, even those overexpressing HER2 (a product of the proto-oncogene erb2). The down-regulation of cyclin D1 mRNA was pre-

Table 3 *Summary of genes up-regulated in both MDA-MB-435 and MCF-7 cells after exposure to CDDO (16 h, 2 μM)*

GenBank accession no.	FC in MCF-7	Gene name	FC in MDA-MB-435
Cell cycle			
U03106	6.5	Cyclin-dependent kinase inhibitor 1A (p21 Cip1)	2.0
L29219	3.4	CDC-like kinase 1	3.8
U61836	2.3	Putative cyclin G1 partial sequence	2.2
Transcription/translation			
U49436	2.1	Eukaryotic translation initiation factor 5, eIf5	2.8
Apoptosis			
AI670788	2.0	Modulator of apoptosis 1	2.4
Transporter			
U70322	2.8	Karyopherin (importin) beta 2	2.1
W28281	17.8	GABA(A) receptor-associated protein like 1	8.1
U83460	2.0	Solute carrier family 31 (copper transporters) member 1	2.6
AB002311	4.4	PDZ domain containing guanine nucleotide exchange factor(GEF) 1	4.7
Transcription factor			
S62138	24.0	TLS/CHOP, Gadd153 member of the C/EBP transcription factor	11.1
X52560	5.5	CCAAT/enhancer binding protein (C/EBP) beta	2.0
U20240	4.6	CCAAT/enhancer binding protein (C/EBP) gamma	2.4
X64318	6.1	E4bp4, a member of the basic region/leucine zipper (bZIP) TF	2.1
S68271	4.8	cAMP responsive element modulator=CREM	5.6
AF096870	6.8	Estrogen-responsive B box protein=B box zinc finger protein family	8.5
M92843	2.4	Zinc finger protein homologous to Zfp-36 in mouse	3.0
AL050162	24.0	Testis derived transcript (3 LIM domains)	3.9
AF012108	2.0	Nuclear receptor coactivator 3, steroid receptor	2.2
Cytoskeleton/cell adhesion			
L78132	6.6	Lectin galactoside-binding soluble 8 (galectin 8)	2.8
W28807	4.6	Microtubule-associated proteins 1A/1B light chain 3	3.1
U32315	4.6	Syntaxin 3A	6.6
U26648	4.1	Syntaxin 5A	2.7
Metabolism			
Z82244	137.4	Heme oxygenase (decycling) 1	25.8
L35546	9.4	Glutamate-cysteine ligase modifier subunit	4.8
M90656	4.2	Glutamate-cysteine ligase catalytic subunit	2.5
U43944	4.3	Malic enzyme 1 NADP(+)-dependent cytosolic	2.0
AL049417	3.5	Dual specificity phosphatase 3 (vaccinia virus phosphatase VH1-related)	2.3
AF061016	3.2	UDP-glucose dehydrogenase	3.6
X91247	2.7	Thioredoxin reductase 1	3.6
X54326	2.6	Glutamyl-prolyl-tRNA synthetase	2.0
M90516	2.4	Glutamine-fructose-6-phosphate transaminase 1	4.9
M77693	2.1	Spermidine/spermine N1-acetyltransferase	3.9
Signal transduction			
Y07566	6.2	Ric (Drosophila)-like expressed in many tissues, Ras-like GTPases	5.1
AC005192	5.9	Interferon-related developmental regulator 1	2.0
X68277	2.5	Dual specificity phosphatase 1	3.5
J03805	2.1	Protein phosphatase 2 (formerly 2A) catalytic subunit beta isoform	2.3
X80692	3.3	Mitogen-activated protein kinase 6	2.2
Proteolysis, ubiquitin-proteasome pathway			
Z29331	3.4	Ubiquitin-conjugating enzyme E2H (homologous to yeast UBC8)	2.4
AF055001		Homocsleine-inducible endoplasmic reticulum stress-inducible ubiquitin-like domain member 1	4.7
U46751	2.9	Sequestosome 1, ubiquitin-binding protein sequestosome 1	2.8
AF012086	2.9	RAN binding protein 2-like 1	2.2
Heat shock protein			
D63861	2.6	Peptidylprolyl isomerase D (cyclophilin D)=heat shock40	2.3
L15189	2.4	Heat shock 70kD protein 9B (mortalin-2)	2.3
X87949	2.3	Heat shock 70kD protein 5 (glucose-regulated protein 78kD)	3.0
M11717	2.0	Heat shock 70 kD protein 1A	19.6
L08069	2.2	DnaJ (Hsp40) homolog subfamily A member 1	3.3
D85429	2.0	DnaJ (Hsp40) homolog subfamily B member 1	6.0
Others			
AF002020	3.0	Niemann-Pick disease type C1	2.0
U78027	2.7	Bruton agammaglobulinemia tyrosine kinase	3.3
AF039103	2.7	HIV-1 Tat interactive protein 2 30 kDa	2.3

vented by pretreatment with actinomycin D, suggesting that cyclin D1 is also regulated at the transcriptional level.

$P21^{Waf1/CIP1}$ inhibits cell cycle progression leading to G_1 arrest. At the molecular level, $P21^{Waf1/CIP1}$ inhibits CDK activity with a certain selectivity for G_1-S-phase cyclin-CDK complexes (62). $p21^{Waf1/CIP1}$ also activates cyclin D-CDK complexes by increasing the stability of the D-type cyclins (63) and by directing these complexes to the cell nucleus (64, 65). In addition to its role in G_1 transition, $p21^{Waf1/CIP1}$ reaccumulates in nuclei near the G_2-M boundary and promotes a transient block late in G_2 (66). In the breast cancer cell lines studied, the up-regulation of $p21^{Waf1/CIP1}$ by CDDO is consistent with the G_1-S arrest and probably also with the G_2-M block observed. The experiments with HCT116 $P21^{Waf1/CIP1}$ −/− cells support the importance of $P21^{Waf1/CIP1}$ in CDDO-mediated cell cycle arrest. The expression of CDK2 and CDK4 remained constant in the presence of CDDO. Taken together, the observed increase in $P21^{Waf1/CIP1}$ and decrease in cyclin D1 suggests that CDDO is a potent inhibitor of cell cycle progression in breast cancer, with concomitant effects on cyclin D1 and $p21^{Waf1/CIP1}$. This conclusion is additionally substantiated by the finding that the *p21* gene contains a potential conserved consensus *PPRE* in the promoter region (67). It has also been observed that $P21^{Waf1/CIP1}$ mediates *p53*-induced cell cycle arrest in cells with DNA damage after irradiation, but $p21^{Waf1/CIP1}$ can be induced independently of *p53* (68). This was reported for a novel alkylphospholipid found to promote cell cycle arrest at either G_1-S or G_2-M (69). To determine the role of p53 in CDDO-mediated cell cycle arrest, we

Table 4 *Differential expression by real-time PCR of genes identified by cDNA arrays in MDA-MB-435 and MCF-7 cells*[a]

Gene	MDA-MB-435 cell line		MCF-7 cell line	
	DMSO	2 μM CDDO	DMSO	2 μM CDDO
Cyclin D1	1	1/13	1	1/6
Cyclin E	1	×1.3	1	1/1.9
p21$^{Waf1/CIP1}$	1	×4	1	×21
p27^{KIP1}	1	×1	1	1/1.8
Bcl-2	1	1/1.3	1	1/20

[a] Cells were exposed to 2 μM CDDO or vehicle for 16 h. mRNA expression was quantified as described in "Materials and Methods." The mRNA expression of each gene in the presence of DMSO was considered the reference value and is denoted by "1" in the table. The mRNA expression in the presence of CDDO is that in comparison with this reference value. *Cyclin D1* mRNA was down-regulated, and *p21*$^{Waf1/CIP1}$ was up-regulated in both cell lines; *Bcl-2* was down-regulated only in MCF-7 cells.

tested cells with different *p53* status (wild type and mutant). Results demonstrated that *p21*$^{Waf1/CIP1}$ was indeed up-regulated by CDDO independent of *p53*. Besides CDK regulation, p21$^{Waf1/CIP1}$ directly binds to PCNA, which is associated with DNA replication and cell proliferation (70). In the growth inhibition of the breast cancer cells produced by CDDO, however, PCNA down-regulation could either indicate the lack of proliferation or be the result of complete inhibition linked to the overexpression of p21$^{Waf1/CIP1}$ (71).

Our findings additionally demonstrate that CDDO induces apoptosis in breast cancer cells by reducing $\Delta\psi_m$, followed by the translocation of phosphatidylserine to the cell surface. In addition, we demonstrate down-regulation of antiapoptotic *Bcl-2* and up-regulation of proapoptotic *GADD153*, also known as *cyclophosphamide-Adriamycin-vincristine-prednisone*, by CDDO by a factor of at least 10. Our group has already reported that CDDO can induce apoptosis in leukemia cell lines and primary AML samples, in part, through down-regulation of Bcl-2 (38). *GADD153* is a member of the C/EBPα family of transcription factors and is transcriptionally activated by a variety of growth arrest and/or damaging factors (72). Several chemotherapeutic drugs induce *GADD153* expression and apoptosis. The

Table 5 *Differential expression of cyclin D1, cyclin E, p21*$^{Waf1/CIP1}$*, p27*^{KIP1}*, Bcl-2, and PPARγ mRNA shown by real-time PCR in MCF-7, MDA-MB-435, and MDA-MB-231 cells at 24, 48, and 72 h in the presence of 1 μM CDDO or vehicle*[a]

Cell line	Cyclin D1	Cyclin E	p21^{Waf1}	p27^{KIP1}	Bcl-2	PPARγ
MCF-7						
24 h	/2.5		×17		/2.8	×2.26
48 h	/5		×40		/3.35	×7
72 h			×43	×3.4		×7.43
MDA-MB-435						
24 h			×33			×2.9
48 h	/17	×2.64	×51			×28
72 h	×2.18		×62		/2.68	×36
MDA-MB-231						
24 h			×21			/2.56
48 h		×2.43	×34			
72 h	/7.9	×4.40	×49	×2.74		

[a] Cells were exposed to 1 μM CDDO or vehicle for 24, 48, and 72 h. mRNA expression was quantified as described in "Materials and Methods." The expression of the mRNA of each gene of interest in the presence of CDDO was compared with that in the presence of DMSO. The table shows those differential mRNA expressions that exceeded 2-fold. *Cyclin D1* mRNA was down-regulated, and *p21*$^{Waf1/CIP1}$ was up-regulated in all three cell lines.

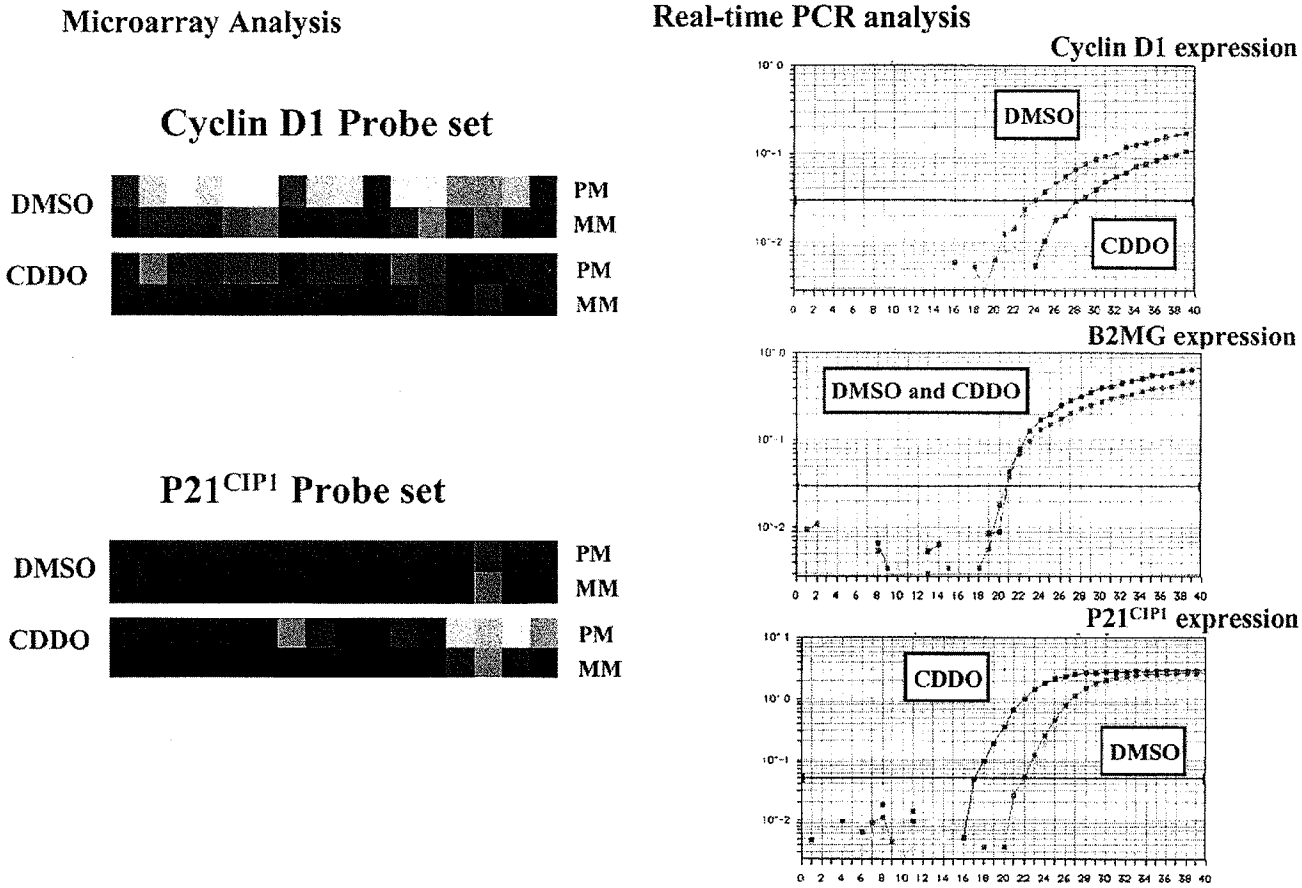

Fig. 6. Microarray and real-time PCR: differential expression of a *cyclin D1* and *P21*$^{WAF1/CIP1}$ in MCF-7 cells treated with CDDO. Cells were grown in the presence of 2 μM CDDO or vehicle for 16 h and analyzed by Affymetrix microarrays and real-time PCR. The *cyclin D1* and *P21*$^{Waf1/CIP1}$ probe sets were composed of 16 probe pairs. A perfect match (p.m.) and mismatch (MM) form each probe pair, and the intensity of the expression of each probe pair was detected. The figure shows changes in intensity for both genes in the presence of CDDO or vehicle alone. The 7-fold down-regulation of *cyclin D1* mRNA and the 6.5-fold up-regulation of *P21*$^{Waf1/CIP1}$ mRNA found by microarray analysis were confirmed by real-time PCR, which showed 6- and 21-fold changes, respectively.

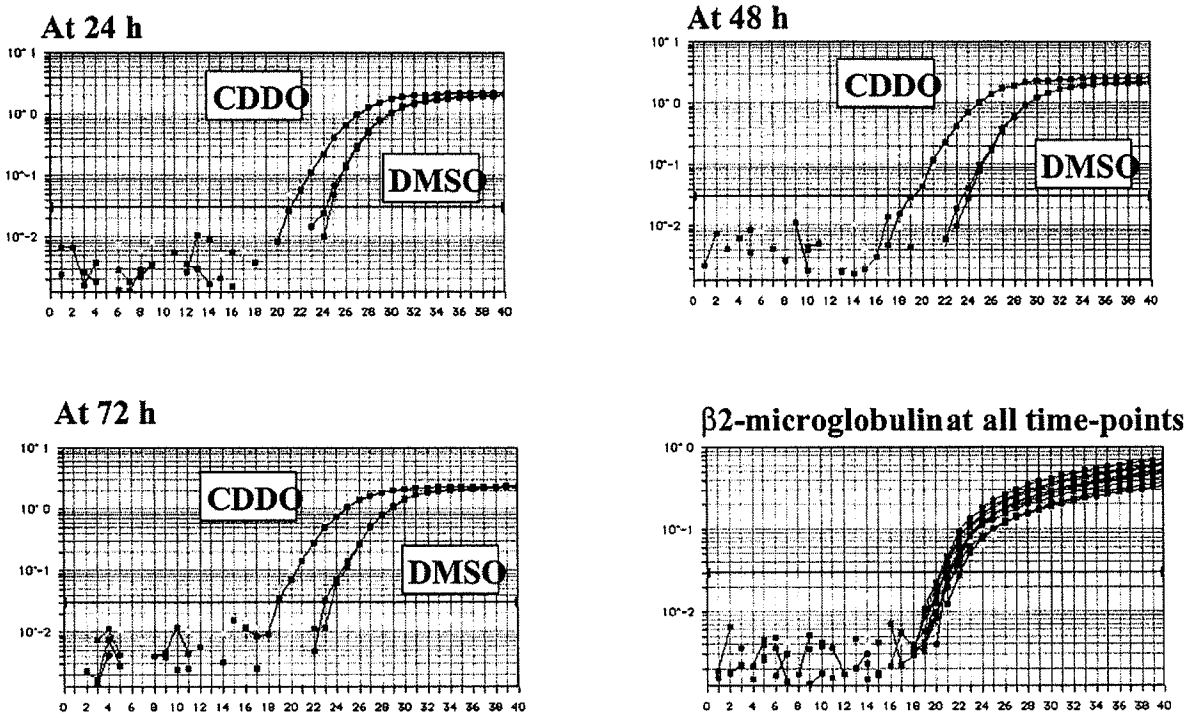

Fig. 7. Time-dependent increase in $p21^{Waf1/CIP1}$ mRNA expression induced by CDDO (1 μM). Sample results in MDA-MB-231 cells. Cells were collected after 24, 48, and 72 h of treatment with CDDO (1 μM) or vehicle alone. RNA extraction and real-time PCR were performed for each time point. The curves depict the differential expression in untreated (DMSO) and CDDO-treated cells. Each sample was normalized to BMG, and results were expressed as FC. In MDA-MB-231 cells, $p21^{Waf1/CIP1}$ mRNA expression was up-regulated 21-fold at 24 h, 34-fold at 48 h, and 49-fold at 72 h.

expression of *GADD153*, in turn, induces growth arrest and apoptosis in M1 myeloblastic leukemia cells (72), neuroblastoma (73), and hepatoma cells (74). Recently, *GADD153* was reported to down-regulate *Bcl-2*, thereby sensitizing cells to endoplasmic reticulum stress (75). The induction of *GADD153* in breast cancer cell lines by CDDO and its potential link to *Bcl-2* down-regulation requires additional investigation.

Other proteins encoded by CDDO targeted genes are already known to be regulated by PPARγ. These include members of the C/EBP transcription factor family and proteins involved in lipid metabolism. Of the six C/EBP proteins, C/EBPβ and C/EBPδ play an important functional role in the mammary gland. C/EBPδ appears to be most important for growth arrest and apoptosis, whereas C/EBPβ is necessary for growth and differentiation (76). PPARγ, C/EBPβ, C/EBPδ, and later C/EBPα appear to be involved in the adipocytic differentiation program (77). In our experiments, CDDO induced the expression of *C/EBPβ* and *C/EBPγ* mRNA, an effect even more pronounced in MCF-7 cells in which adipocyte-like differentiation was observed (7).

In conclusion, our data provide the first evidence that CDDO, a PPARγ ligand, induces cell growth inhibition, cell cycle arrest, and apoptosis by targeting important genes involved in human breast carcinogenesis. Cyclin D1 emerges as a major target of CDDO because of the direct relationship between its overexpression and murine

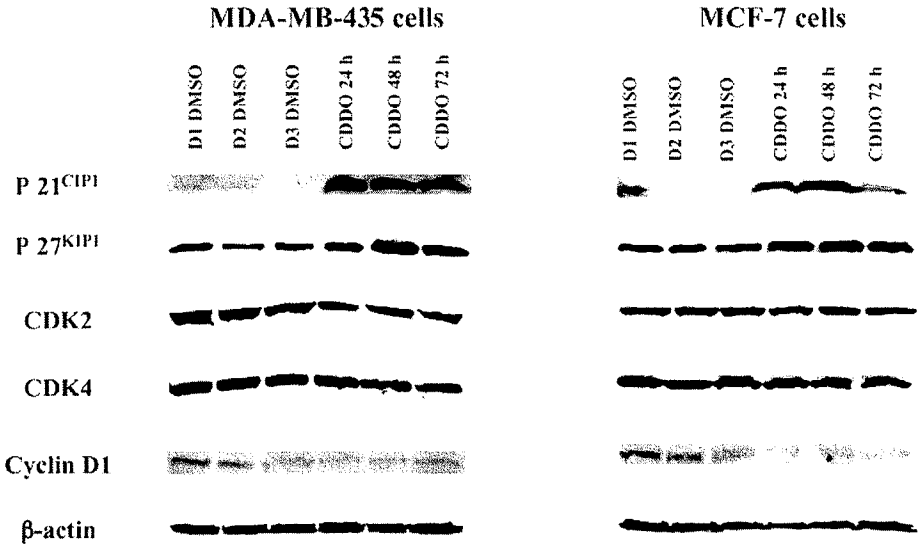

Fig. 8. Expression of cyclin D1, CDK2, CDK4, $P21^{Waf1/CIP1}$, and $p27^{KIP1}$ in breast cancer cell lines in the presence of 1 μM CDDO, determined by Western blot analysis. MDA-MB-435 and MCF-7 cells were exposed to 1 μM CDDO or vehicle and were collected at 24, 48, and 72 h. Each sample was processed for Western blotting as described in "Materials and Methods." Western blot analysis confirmed results shown by microarray and real-time PCR at the protein level with down-regulation of cyclin D1 and up-regulation of $p21^{Waf1/CIP1}$. Of interest, the expression of $p27^{KIP1}$ appeared to increase 2-fold. The expression levels of the CDK2 and CDK4 kinases were constant during the entire experiment.

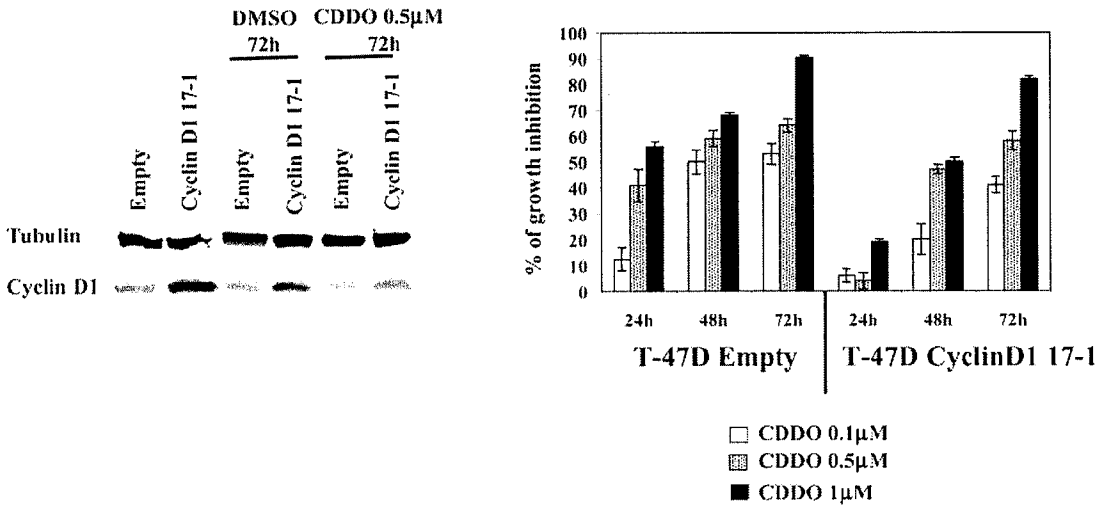

Fig. 9. Growth inhibition of T-47D breast cancer cells with constitutive cyclin D1 overexpression. Western blot showed 5-fold increased expression of cyclin D1 in T-47D-cyclin D1 17-1 compared with control (T-47D-empty) cells. Cells were incubated with different concentrations (0.1, 0.5, and 1 μM) of CDDO or DMSO for 24, 48, and 72 h. The *bar graphs* show normalized growth inhibition as determined by cell counts. Effects of CDDO on cyclin D1 expression were analyzed by Western blot (0.5 μM CDDO, 72 h).

or human breast cancer (4, 60, 61) and because of the resistance of cyclin D1-deficient mice to mammary carcinomas induced by *c-neu*. In the same vein, strategies targeting insulin growth factor 1 receptor signaling may prevent or delay the development of resistance to trastuzumab, an anti-HER2 antibody used in the treatment of breast cancers overexpressing HER2 (78). We demonstrate here that CDDO decreased the expression of cyclin D1 and insulin receptor substrate 1 in all breast cancer cell lines studied, independent of their ER and HER2 status. We are currently investigating the potential effect of CDDO on the HER2 signaling pathway. We additionally established effects of CDDO on the expression of $p21^{Waf1/CIP1}$, *PCNA*, *Bcl-2*, and *GADD153*, which are all consistent with the growth inhibition, cell cycle arrest, and induction of apoptosis observed in breast cancer cell lines. Furthermore, CDDO inhibited breast cancer growth *in vivo* in immunodeficient mice and our preliminary data demonstrate that CDDO is also able to abrogate growth of MCF-7/HER2 tumors in an immunodeficient xenograft model (79). In pharmacokinetic studies conducted at M. D. Anderson Cancer Center, after a single i.v. dose of CDDO at 30 mg/kg, mean peak concentrations of 2.0 ± 0.8 mg/ml were achieved (4.1 ± 1.6 μM; Ref. 80). In the study reported here, complete cytostatic and apoptotic effects were achieved *in vitro* at 1 μM CDDO, suggesting that effective concentrations can be achieved *in vivo*. These collective findings show the potential value of PPARγ ligation by CDDO as novel treatment strategy in human breast cancer.

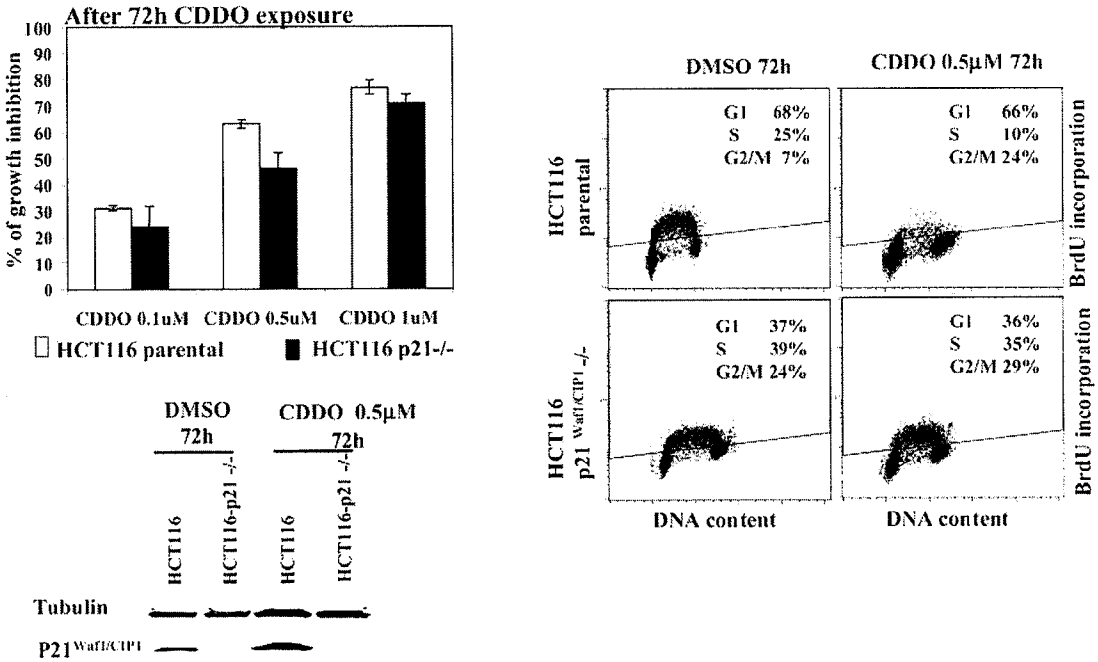

Fig. 10. Effect of CDDO on growth inhibition and cell cycle distribution of parental and $p21^{Waf1/CIP1}$-knockout HCT116 cells. *Bar graphs* demonstrate the relative growth inhibition at 72 h, as determined by cell counts. The expression of $p21^{Waf1/CIP1}$ was determined by Western blot analysis; tubulin expression was used as loading control. The percentages of cells in each phase of the cell cycle were calculated by double fluorescence BrdUrd/PI fluorescence-activated cell sorting analysis. CDDO inhibited proliferation in the parental cell line, as shown by a decrease in cells in the S phase and an accumulation in G_2-M but not in HCT116 $p21^{Waf1/CIP1}$ −/− cells.

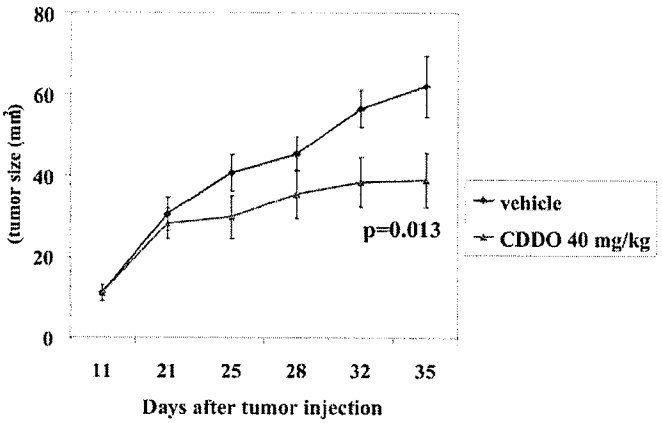

Fig. 11. Effect of CDDO on breast cancer *in vivo*. Female nude immunodeficient mice (n = 25) were inoculated s.c. with 2 × 10^6 MDA-MB-435 cells/mouse. Ten days after inoculation, 10 mice were treated with CDDO (40 mg/kg i.v. twice a week for 3 weeks). Fifteen mice received vehicle only. The tumor was measured twice weekly with microcalipers, and the tumor volume was calculated as the length × width (in millimeters). Results demonstrate a statistically significant reduction in tumor growth in the treated group compared with the vehicle group (P = 0.013).

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2726

Synthetic triterpenoid CDDO as a novel therapy for resistant breast cancer

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We have studied the effects of novel triterpenoid CDDO (2-cyano-3,12-dioxoolen-1,9-dien-28-oic acid) on the proliferation and apoptosis of breast cancer cells. In all breast cancer cell lines studied, CDDO induced cell growth inhibition in a dose- and time-dependent manner, cell cycle arrest in the G1/S and G2/M phases, and apoptosis. These effects are independent of the estrogen receptor or p53 status. We utilized differential cDNA array analysis to investigate the molecular changes induced by CDDO. After a 16-h exposure of MCF-7 and MDA-MB-435 cells to CDDO, we found the genes that encode the following proteins to be upregulated in both cell lines: p21Waf1/CIP1, GADD153, members of the C/EBP transcription factor family, and proteins involved in the ubiquitin-proteasome pathway. Among the downregulated genes, we especially focused on the genes encoding cyclin D1, PCNA, and the insulin receptor substrate 1. Using western blot analysis and/or real-time PCR, we confirmed that CDDO regulated the expression of cyclin D1, p21Waf1/CIP1, and Bcl-2. In a murine breast cancer xenograft model using ER(-) MDA-MB-435 cells, treatment with CDDO at 40mg/kg IV twice per week induced significant reduction in tumor growth compared to the vehicle control group (p=0.03). Since overexpression of human epidermal growth factor receptor (HER2) translates into aggressive disease in patients with breast cancer, we compared effects of CDDO in HER2-transfected MDA-MB-435 and MCF-7 cells. 1[mu]M CDDO completely abrogated growth of HER2-expressing cells similar to vector-transduced cells. We found that CDDO downregulated HER2 expression and phosphorylation as determined by Western blot analysis and phosphotyrosine phosphorylation of immunoprecipitated HER2 protein.
In summary, CDDO induces growth arrest and apoptosis in breast cancer cells via targeting genes involved in the regulation of the cell cycle and apoptosis. These data indicates the usefulness of CDDO as a novel therapy in breast cancer including ER(-) and HER2(+) cancers.

Synthetic triterpenoid CDDO as effective therapy for HER2-expressing resistant breast cancer

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Overexpression of the HER2 proto-oncogene confers resistance of breast cancer cells to antiestrogens and chemotherapy and translates into aggressive disease with short survival. Despite clinical benefit of the HER2 inhibitor Trastuzumab in metastatic breast cancer with overexpressed HER2, most patients are developing resistance. We have reported that the novel synthetic triterpenoid CDDO (2-cyano-3,12- dioxooleana-1,9-dien-28-oic acid) induces growth arrest and apoptosis in breast cancer cells. We studied the effects of CDDO on proliferation and differentiation of breast cancer cell lines with different HER2 expression levels including stably transfected MCF7/HER2 and MDA-MB-435/HER2 cells, vector controls and SKBR3 cells with constitutive HER2 overexpression. Results demonstrate dose-dependent inhibition of colony formation by CDDO at submicromolar concentrations, with HER2-overexpressing cells showing increased sensitivity. CDDO induced adipocytic differentiation of breast cancer cells, as determined by Oil Red-O staining. Concomitantly, proliferation was inhibited independent of HER2 levels. CDDO arrested both MCF7/HER2 and MCF7/neo cells in G1 phase and prevented exit from G1 in synchronized cells. Furthermore, CDDO downregulated cyclin D1 and B1 mRNA and protein expression in both, vector- and HER2-transfected cells, and reduced cyclin A and cdc2 protein levels without change in cyclin E. These effects were more pronounced in HER2-overexpressing breast cancer cells compared with vector controls. We further demonstrated that CDDO downregulated HER2 protein levels in a dose- and time-dependent fashion. No changes in HER2 mRNA expression were noted suggesting post-transcriptional mechanisms. HER2 downregulation was partially inhibited by proteasome inhibitors MG132, PSI and calpeptin. In addition, CDDO inhibited HER2 auto- and transphosphorylation of enolase in *in vitro* phosphorylation assays. To test anti-tumor effects of CDDO *in vivo*, a murine breast cancer xenograft model was established by subcutaneous injection of MCF7/Neo, MCF7/HER2 or MDA-MB-361 cells into nude/nude mice. At the start of therapy, MCF7/HER2 tumors were twice as large as MCF7/Neo and continued to grow faster. Remarkably, liposomally encapsulated CDDO at 20 mg/kg/day IV abrogated tumor growth in both tumor types equally. Furthermore, CDDO decreased HER2 expression in tumors as determined by histochemistry, confirming the *in vitro* data.

These findings provide the first evidence that CDDO inhibits breast cancer growth *in vitro* and *in vivo* by mechanism(s) independent of HER2 and suggest utility of CDDO for the treatment of HER2-overexpressing breast cancer.

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Growth-inhibitory effect of a novel synthetic triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, on ovarian carcinoma cell lines not dependent on peroxisome proliferator-activated receptor-γ expression

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Abstract

Objectives. Despite the advent of new chemotherapeutic drugs in recent decades, epithelial ovarian carcinoma (EOC) remains the leading cause of death from gynecologic cancers, and new therapeutic targets and agents are urgently needed. 2-Cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) is a novel synthetic triterpenoid with anti-tumor activity against a wide range of tumors in vitro and in vivo. CDDO is a ligand for the peroxisome proliferator-activated receptor-γ (PPARγ). The aim of the present study was to evaluate CDDO activity in EOC cell lines in vitro.

Methods. The expression of PPARγ was examined by real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) in eight EOC cell lines (2774, SKOV3, CAOV3, OVCAR3, NMP-1, HEY, 2008 and 2008.C13), and the growth inhibitory activity of CDDO was assessed using the MTT assay.

Results. PPARγ RNA was expressed in all eight cell lines examined, but the expression varied widely among cell lines. In contrast, CDDO showed a similar degree of activity in different EOC cell lines independent of cisplatin sensitivity, with 50% inhibitory concentrations ranging from 1 to 4 μM. Experiments combining CDDO with cisplatin and paclitaxel indicated weak antagonism. The growth-inhibitory activity of CDDO was unaffected by

PPARγ antagonist T007.

Conclusions. Although differences were observed in PPARγ expression in EOC cell lines, CDDO had similar growth-inhibitory activity in all cell lines examined, indicating that the antitumor activity of CDDO in vitro is mediated by a mechanism independent of PPARγ. The activity of CDDO in platinum-resistant cell lines is encouraging with respect to the potential clinical use of the drug.

Author Keywords: Author Keywords: 2-Cyano-3,12-dioxoolean-1,9-dien-28-oic acid; Epithelial ovarian carcinoma; Peroxisome proliferator-activated receptor-γ

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Selected Abstracts

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Sudhakar Chintharlapalli, Sabitha Papineni, Marina Konopleva, Michael Andreef, Ismael Samudio, and Stephen Safe

2-Cyano-3,12-dioxoolean-1,9-dien-28-oic Acid and Related Compounds Inhibit Growth of Colon Cancer Cells through Peroxisome Proliferator-Activated Receptor γ-Dependent and -Independent Pathways

Mol. Pharmacol. 68: 119-128.

Abstract 1 of 1

2-Cyano-3,12-dioxoolean-1,9-dien-28-oic Acid and Related Compounds Inhibit Growth of Colon Cancer Cells through Peroxisome Proliferator-Activated Receptor γ-Dependent and -Independent Pathways

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2-Cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) and the corresponding methyl (CDDO-Me) and imidazole (CDDO-Im) esters induce peroxisome proliferator-activated receptor γ (PPARγ)-dependent transactivation in SW-480 colon cancer cells, and these responses were inhibited by small inhibitory RNA for PPARγ. Moreover, in a mammalian two-hybrid assay using the PPARγ$_2$-VP16 fusion plasmid and GAL4-coactivator/corepressor chimeras and a construct (pGAL4) containing five tandem GAL4 response elements, CDDO, CDDO-Me, and CDDO-IM induce transactivation and PPARγ interaction with multiple coactivators. A major difference among the three PPARγ agonists was the higher activity of CDDO-Im to induce PPARγ interactions with the corepressor SMRT. CDDO, CDDO-Me, and CDDO-Im inhibited SW-480, HCT-116, and HT-29 colon cancer cell proliferation at low concentrations and induced cell death at higher concentrations. Growth inhibition at lower concentrations correlated with induction of the tumor suppressor gene caveolin-1 which is known to inhibit colon cancer cell growth. Induction of caveolin-1 by CDDO, CDDO-Me, and CDDO-Im was inhibited by the PPARγ antagonist *N*-(4'-aminopyridyl-2-chloro-5-nitrobenzamide (T007), whereas higher doses induced apoptosis [poly(ADP-ribose) polymerase cleavage], which was not inhibited by T007. These results illustrate that CDDO-, CDDO-Me, and CDDO-Im induce both PPARγ-dependent and -independent

responses in colon cancer cells, and activation of these pathways are separable and concentration-dependent for all three compounds.

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96th Annual Meeting
April 16-20, 2005
Anaheim/Orange County, CA

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Abstract Number: 5899

Presentation Title: 2-cyano-3,12 dioxoolean-1,9 diene-28-imidazolide induces apoptosis in pancreatic cancer via redox-dependent cytoplasmic stress

Presentation Start/End Time: Wednesday, Apr 20, 2005, 8:00 AM -12:00 PM

Board Number: Board #19

Author Block: *Ismael Samudio, Marina Konopleva, Numsen Hail Jr., Yue-Xi Shi, Teresa McQueen, Timothy Hsu, Randall Evans, Michael Andreeff.* UT M. D. Anderson Cancer Center, Houston, TX, The University of Texas at Austin, Austin, TX

Surgical resection is the only curative strategy for pancreatic cancer (PC). Unfortunately, over 80% of pancreatic cancer patients bear inoperable, locally advanced, chemoresistant tumors demonstrating the urgent need for development of novel therapeutic approaches to treat this disease. Here we report that the novel triterpenoid 2-cyano-3,12 dioxoolean-1,9 diene-28-imidazolide (CDDOim) induces apoptosis in PC cells in a dose- and time-dependent manner abrogating cell growth at submicromolar concentrations. Our results demonstrate that CDDOim induces phosphatidyl serine (PS) externalization, loss of mitochondrial membrane potential (MMP), activation of caspases, generation of reactive oxygen species (ROS), increases in intracellular free calcium, and rapid increase in oxidized glutathione levels. Depletion of intracellular glutathione or NADPH exacerbated the cytotoxicity of CDDOim, while cotreatment with sulfhydryl antioxidants completely prevented apoptosis and loss of viability indicating that intracellular reduced thiols are functional targets of this synthetic triterpenoid. Notably, treatment of PC cells with CDDOim resulted in 30-40% inhibition of thioredoxin reductase (TR) activity without affecting thioredoxin (Trx) or glutathione reductase (GR) activities suggesting that TR is one of the critical redox-regulating targets of CDDOim. Consistent with inhibition of TR activity, we observe p38 MAPK upregulation of heme oxygenase -1 (HO-1), and p38 MAPK-dependent activation of caspases. Interestingly, CDDOim induced rapid morphological changes reminiscent of apoptosis but occurring before membrane potential loss, and annexin positivity. Immunohistochemical analysis of α-tubulin in CDDOim treated cells revealed a marked disorganization of the cytoskeleton, suggesting that inhibition of TR activity contributes to oxidation of critical cytoskeletal proteins. Finally, pharmacological inhibition of caspases or p38 MAPK failed to prevent the loss of viability or the morphological changes induced by CDDOim. Taken together, our observations demonstrate that CDDOim abrogates the growth of PC cells by inducing redox-dependent, caspase-independent apoptosis and/or necrosis. Ongoing studies are aimed at elucidating the contributions of TR inhibition to CDDOim induced apoptosis, and identifying additional redox-modulating targets of this synthetic triterpenoid.

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Synthetic triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid induces growth arrest in HER2-overexpressing breast cancer cells

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Abstract

HER2 overexpression is one of the most recognizable molecular alterations in breast tumors known to be associated with a poor prognosis. In the study described here, we explored the effect of HER2 overexpression on the sensitivity of breast cancer cells to the growth-inhibitory effects of 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), a synthetic triterpenoid, both *in vitro* and *in vivo* in a xenograft model of breast cancer. Both cell growth and colony formation in the soft agar assay, a hallmark of the transformation phenotype, were preferentially suppressed in HER2-overexpressing cell lines at low concentrations of CDDO, whereas growth-inhibitory effects at high concentrations did not correlate with the expression level of HER2. CDDO dose-dependently inhibited phosphorylation of HER2 in HER2-overexpressing cells and diminished HER2 kinase activity *in vitro*. CDDO induced the transactivation of the nuclear receptor peroxisome proliferator-activated receptor-γ in both vector control and HER2-transfected MCF7 cells. Dose-response studies showed that the growth inhibition seen at lower concentrations of CDDO correlated with induction of the tumor suppressor gene caveolin-1, which is known to inhibit breast cancer cell growth. CDDO also reduced cyclin D1 mRNA and protein expression. *In vivo* studies with liposomally encapsulated CDDO showed complete abrogation of the growth of the highly tumorigenic MCF7/HER2 cells in a xenograft model of breast cancer. These findings provide the first *in vitro* and *in vivo* evidence that CDDO effectively inhibits HER2 tyrosine kinase activity and potently suppresses the growth of HER2-overexpressing breast cancer cells and suggest that CDDO has a therapeutic potential in advanced breast cancer. [Mol Cancer Ther 2006;5(2):317–28]

Introduction

One of the most common molecular alterations associated with the malignant phenotype of breast cancer is the overexpression of epidermal growth factor receptor-2 (also known as HER2). The HER2 receptor is the protein product of the *HER2* proto-oncogene and a member of the epidermal growth factor receptor family of transmembrane tyrosine kinases. Overexpression of HER2 can transform normal mammary epithelial cells and is amplified in 25% to 30% of breast cancers; it is associated with an aggressive form of the disease characterized by significantly shortened survival times (1).

Multiple lines of experimental evidence suggest that the overexpression of HER2 confers antiestrogen resistance on breast tumor cells (2). HER2 overexpression also confers resistance to paclitaxel (Taxol; ref. 3) and alkylating agents (cisplatin and cyclophosphamide; ref. 1). Trastuzumab, a recombinant monoclonal antibody against HER2, has shown some clinical benefit in HER2-overexpressing metastatic breast cancer. However, only a modest response to this highly selective inhibitor was seen when it was given as a single agent, with most cancers becoming resistant to the agent within <12 months of the start of therapy (4). Therefore, it is imperative to identify new agents that can arrest cell growth or induce apoptosis in HER2-overexpressing resistant breast cancer cells.

The novel triterpenoid 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) is effective in inducing apoptosis in leukemic (5–7), multiple myeloma (8, 9), lung cancer (10), ovarian cancer (11), osteosarcoma (12), and melanoma (13) cells. CDDO reportedly binds to and transactivates the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ; ref. 14), a transcription factor that controls key differentiation genes. PPARγ ligands have been reported to inhibit the proliferation of malignant cells from different tissues, such as liposarcoma and breast, prostate, colon,

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non–small cell lung, pancreatic, bladder, and gastric carcinoma cells (15, 16). This effect has been linked to the inhibition of G_0-G_1-S phase cell cycle progression, down-regulation of cyclin D1, enhanced expression of p21 or p27 cyclin-dependent kinase inhibitors, and induction of apoptosis. Activation of PPARγ by 15-deoxy-Δ12,14-prostaglandin J2 was also reported to dramatically inhibit HER2 tyrosine phosphorylation, which resulted in cell growth suppression and apoptosis (17). PPARγ ligands also inhibit growth of PPARγ-deficient cells (18), and PPARγ-independent responses have been reported in several cancer cell lines (19–21).

We have shown previously that CDDO induces apoptosis in leukemic cells (22, 23) and inhibits the proliferation of both estrogen receptor (ER)–positive and ER-negative breast cancer cells (24) in part by activating PPARγ signaling. However, CDDO inhibited growth of ovarian cancer cells irrespective of the PPARγ status of the cells (11). In this study, we investigated the effects of CDDO on HER2-overexpressing breast cancer cells and the mechanisms of these responses. We observed that CDDO is capable of inhibiting growth of HER2-overexpressing cells, both *in vitro* and in a xenograft murine model of breast cancer, in part via the PPARγ-dependent induction of caveolin-1 expression. These results indicate that CDDO may be useful as an adjunct or alternative therapy to conventional chemotherapy for chemoresistant breast cancer.

Materials and Methods

Chemicals, Antibodies, and Other Materials

CDDO was manufactured through the NIH Rapid Access to Interventional Development Program and kindly provided by Drs. E. Sausville (Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD) and M. Sporn (Department of Pharmacology, Dartmouth Medical School, Hanover, NH). A stock solution of 10 mmol/L CDDO in DMSO was kept stored at −20°C.

Proteasome inhibitors MG132 and proteasome inhibitor I were purchased from Calbiochem Co. (San Diego, CA). [γ-^{32}P]ATP and [^{32}P]dCTP were purchased from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom). Cyclin D1 and β_2-microglobulin primers and probes were described by us previously (24). HER2 primers (forward 5′-CCTGCCAGTCCCGAGACCCACCT-3′ and reverse 5′-TTGGTGGGCAGGTAGGTGAGTT-3′) were synthesized by Sigma Genosys (The Woodlands, TX). Glyceraldehyde-3-phosphate dehydrogenase probe was kindly provided by Dr. Duen-Hwa Yan (Department of Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX). Mouse monoclonal antibody HER2/ErbB2 and rabbit polyclonal antibody phospho-HER2 were from Cell Signaling Technology (Beverly, MA); cyclin D1 and cyclin E monoclonal antibodies from Calbiochem; mouse monoclonal antibody PPARγ, rabbit polyclonal phosphotyrosine, and caveolin-1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); and mouse monoclonal antibody ER-α was from DakoCytomation (Glostrup, Denmark). Terminal deoxynucleotide transferase–mediated dUTP nick end labeling (TUNEL) kit was purchased from Roche Diagnostics Corp. (Mannheim, Germany). All of the other chemicals and solvents were of the highest grade commercially available.

Cell Lines and Cell Culture

Breast cancer cell lines that express different levels of HER2, including stably transfected MCF7/HER2 and MDA-MB-435/HER2 cells and their vector controls (25) and SKBR3 cells that constitutively overexpress HER2 (a gift from Dr. Xiaofeng Le, Department of Experimental Therapeutics, The University of Texas M. D. Anderson Cancer Center), were used as *in vitro* model systems. The cells were cultured in DMEM/F12 supplemented with 10% (v/v) FCS and L-glutamine. Cells were maintained at 37°C in an atmosphere of 5% CO_2-95% air.

Growth Viability Assay

Effects of cell growth were assessed by using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay (Promega, Madison, WI). This assay, a colorimetric method for determining the number of viable cells, is based on the bioreduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt by cells to a formazan product that is soluble in tissue culture medium and can be detected spectrophotometrically. MCF7/Neo, MCF7/HER2, MDA-MB-435/Neo, and MDA-MB-435/HER2 cells were plated in 96-well flat-bottomed plates (Corning, Inc., Corning, NY). The seeded number of cells was 2,000 per well, which was required to obtain an absorbance of ~1.0 to 1.5 at a wavelength of 490 nm, the linear range of the assay, after 72 hours of growth (determined empirically). After overnight incubation, cell culture media were replaced with freshly prepared DMEM/F12 containing 10% FCS and indicated concentrations of CDDO (1, 2.5, and 5 μmol/L) and DMSO for 72 hours of incubation. All experimental points were set up in six wells, and all experiments were repeated at least twice. The data were analyzed by ANOVA using Statistica version 6.1 (StatSoft, Inc., Tulsa, OK). IC_{50}s were generated based on the absorbance values using CalcuSyn version 1.2 (BioSoft, Inc., Ferguson, MO).

Colony Formation in Soft Agarose

Cells (1,000 per well) were seeded in 24-well plates in culture medium containing 0.35% low-melting agarose over a 0.7% agarose base layer in the presence of different concentrations of CDDO or vehicle and incubated for 14 days at 37°C in a humidified 95% O_2-5% CO_2 atmosphere in DMEM/F12 supplemented with 10% FCS and L-glutamine. Colonies were then stained with *p*-iodonitrotetrazolium violet (1 mg/mL stock diluted at 1:500) for 16 hours, and colonies larger than 100 μm in diameter were counted under the Leica stereofluorescence microscope MZFL III (Leica Microsystems, Dallas, TX). Each determination was done thrice.

Western Blotting and Immunoprecipitation Studies

For Western blot analysis, an equal amount of cell lysate (30–60 μg/well, equivalent of 2×10^5–4×10^5 cells) was

separated by 10% to 12% SDS-PAGE, which was followed by immunoblotting onto Hybond-P membranes (Amersham Pharmacia Biotech). After blotting in TBST (50 mmol/L Tris-HCl, 150 mmol/L NaCl containing 0.1% Tween 20) with 5% nonfat milk for 1 hour, the membranes were incubated with primary antibodies overnight at 4°C and then with horseradish peroxidase–conjugated secondary antibody for 1 hour at room temperature. Proteins were visualized using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

For the immunoprecipitation studies, 1 mg total protein from cell lysates was incubated overnight with HER2/ErbB2 primary antibody at 4°C. Protein A/G Plus-agarose (Santa Cruz Biotechnology; 20 μL of 50% bead slurry) was then added, and the mixture was gently rocked for 2 hours at 4°C. The precipitates were next washed four times with ice-cold lysis buffer, resuspended in 3× Laemmli sample buffer, resolved by SDS-PAGE, and immunoblotted with HER2, phospho-HER2, or phosphotyrosine antibodies.

In vitro Kinase Assays

The HER2 *in vitro* kinase reaction was done as described previously (26), with minor modifications. Briefly, MCF7/HER2 cells were lysed in lysis buffer [20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na_3VO_4, 1 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride]. Total protein lysate (1.5 mg) was precipitated, as described above, with the HER2 antibody (4 μL) for a 1-hour rotation at 4°C, after which 30 μL protein A/G Plus-agarose beads was added for an overnight incubation at 4°C. Immunoprecipitates were washed thrice with buffer [50 mmol/L Tris-HCl, 0.5 mol/L LiCl (pH 7.5)] and once with kinase assay buffer [50 mmol/L Tris-HCl (pH 7.5), 10 mmol/L $MnCl_2$]. Kinase activity was tested by adding 10 μCi [γ-^{32}P]ATP (specific activity, 3,000 Ci/mmol; Amersham Pharmacia Biotech), 10 μL enolase (stock solution at 2.5 mg/mL), and 10 μmol/L cold ATP (Sigma Chemical Co., St. Louis, MO) in the presence or absence of the indicated concentrations of CDDO. After 20-minute incubation at room temperature, the reaction was stopped by adding 6× Laemmli buffer and heating at 95°C for 5 minutes. Kinase products were resolved by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and exposed to X-ray film. The same nitrocellulose membrane was then rinsed and subjected to a HER2 immunoblotting procedure as described above.

Transient Transfection and Luciferase Activity Assay

One day before transfection, MCF7 cells were seeded in 12-well plates at a density of 0.5×10^5/mL with DMEM/F12 with 5% fetal bovine serum. Subconfluent cells were transfected with PPREx3-TK-LUC reporter (1 μg; kindly provided by Dr. Ronald M. Evans, The Salk Institute, La Jolla, CA; ref. 27) and SV-β-galactosidase DNA (0.2 μg; Promega) using the Fugene-6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) following the manufacturer's instructions. After 24 hours, transfected cells were treated with 2 μmol/L CDDO or 10 μmol/L ciglitazone (Cayman Chemical, Ann Arbor, MI) or with vehicle for 24 hours. Cells were lysed with 200 μL of 1× reporter lysis buffer and cell extracts were subjected to luciferase and β-galactosidase assay. Luciferase activities were normalized to β-galactosidase activity. Each experiment was repeated three to five times.

Quantitative Real-time Reverse Transcription-PCR

Total RNAs were prepared using TRIzol reagent as described by the manufacturer (Life Technologies, Gaithersburg, MD). Total RNA (1 μg) was reverse transcribed by avian myeloblastosis virus reverse transcriptase (Roche Diagnostic Corp., Chicago, IL) under standard conditions. Duplicate samples of 1 μL of each cDNA were amplified by PCR in the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). The amplification reaction mixture (25 μL) contained cDNAs, cyclin D1 primers and probe, and Taqman Universal PCR Master Mix (PE Applied Biosystems). β_2-Microglobulin was coamplified as an internal control to normalize for variable amounts of cDNA in each sample. The thermocycler variables were as follows: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Results were collected and analyzed to determine the PCR cycle number that generated the first fluorescence signal above a threshold [threshold cycle (C_T); 10 SDs above the mean fluorescence generated during the baseline cycles], after which a comparative C_T method was used to measure relative gene expression. The following formula was used to calculate the relative amount of the transcript of interest in the treated sample (X) and the control sample (Y), both of which were normalized to an endogenous reference value (β_2-microglobulin): $2^{-\Delta\Delta CT}$, where ΔC_T is the difference in C_T between the gene of interest and β_2-microglobulin, with the $\Delta\Delta C_T$ for sample $X = \Delta C_T(X) - \Delta C_T(Y)$.

Northern Blot Analysis

Cells that had been subjected to indicated concentrations of CDDO were lysed in TRIzol, and the total cellular RNA was isolated. The HER2 probe was obtained by cloning the PCR products of amplified cDNA using a pair of HER2 primers. The glyceraldehyde-3-phosphate dehydrogenase probe was used as a loading control. The probes were radiolabeled with [^{32}P]dCTP using the Megaprime DNA Labeling System (Amersham Pharmacia Biotech). Total RNA (10 μg) was denatured and run for 8 hours on a 1% formamide agarose gel at 30V. After staining in ethidium bromide, RNA was transferred to Hybond-N$^+$ membranes (Amersham Pharmacia Biotech). Hybridization was carried out overnight at 68°C in ExpressHyb hybridization solution (Clontech Lab, Chicago, IL). The membranes were washed under highly stringent conditions (0.01× SSC and 0.1% SDS for 30 minutes at 65°C) and exposed at −80°C with a double screen to X-ray films (Kodak, Rochester, NY).

Xenograft Studies in Nude Mice

Five-week-old female nude mice (Harlan Sprague-Dawley, Madison, WI) were housed in the barrier and fed with autoclaved diet without phytoestrogens. Animals first implanted with 0.72 mg, 60-day release, 17β-estradiol pellets (Innovative Research, Sarasota, FL). The next day, 5×10^6 MCF7/Neo or MCF7/HER2 cells suspended

in 300 μL growth factor–reduced Matrigel (BD Biosciences, Bedford, MA) were injected s.c. in the right flank of each animal. Treatment with CDDO or the liposomal formulation alone (i.e., "empty liposomes") was initiated once the tumors reached a volume of ≥300 mm^3.

CDDO was formulated in liposomes at a concentration of 2 mg/mL as follows. First, CDDO was solubilized in *t*-butanol at 37°C at a concentration of 2 mg/mL. Phospholipid distearoyl phosphatidylcholine was solubilized in *t*-butanol at 55°C at a concentration of 10 mg/mL. Distearoyl phosphatidylcholine and CDDO were then mixed together and frozen by being placed at an angle in an acetone and dry ice bath and then quickly turned until the samples were frozen. Samples were then placed on a lyophilizer and freeze-dried overnight. The lipid-to-drug ratio we used was 10:1, 20:1, or 40:1. Empty liposomal controls were made using the same lipids but without CDDO. Liposomal CDDO was reconstituted in normal saline at 55°C to form liposomes and then centrifuged at 13,000 rpm for 1 hour. Pellets were resuspended at room temperature in normal saline at a concentration of 2 mg/mL (100 μmol/L) for the *in vivo* studies. Liposomal CDDO preparations were centrifuged at 13,000 rpm for 1 hour, and pellets were obtained to determine encapsulation efficiency by liquid chromatography-tandem mass spectrometry. Sample were run on the MicroMass Quattro Ultima (tandem mass spectrometer), and CDDO content in the liposomal CDDO pellets was calculated using the neat standard curve with a quantitation range of 5 to 1,000 ng/mL. Analysis of liposomal preparation pellets (four independent experiments) showed that CDDO was incorporated into distearoyl phosphatidylcholine liposomes at or near the target concentration (range, 83–136 μmol/L).

Liposomal CDDO was given i.v. thrice weekly via tail vein injection at a final dose 20 mg/kg/mouse/d (0.4 mg/mouse/d in 200 μL) for 3 weeks. Tumor diameters were serially measured with calipers, and tumor volumes were calculated by the formula: (width^2 × length) / 2.

Histology and Immunohistochemistry Analyses

For the histologic analysis, excised tumors were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with H&E. Additional sections were used for immunostaining with cyclin D1 and phospho-HER2 antibodies. Briefly, antigen retrieval was achieved by heating the sections in 10 mmol/L citrate buffer (pH 6.5) in a microwave oven for 20 minutes. Before the sections were stained, endogenous peroxidase was quenched using 3% H_2O_2, and nonspecific binding was blocked using 10% normal goat serum incubated for 1 hour at room temperature. The slides were then incubated with the indicated antibodies overnight at 4°C. The immunostaining was visualized by the avidin-biotin-peroxidase complex method using a Vectastain Elite ABC kit and 3-amino,9-ethyl-carbazole (Vector Laboratories, Burlingame, CA) as chromogen. Slides were counterstained with Mayer's hematoxylin (Fisher Scientific International, Inc., Pittsburgh, PA). The slides were analyzed under a Nikon Optiphot microscope with a digital capture camera (Microscopy Documentation System 290, Eastman Kodak, New Haven, CT), and the mean number ± SD of positive cells was counted from three to five random areas of ×20 field for each slide.

TUNEL Assay

For the *in situ* detection of apoptotic cells in xenograft tumor tissues, a TUNEL assay was done using TUNEL kit (Roche Diagnostics, Mannheim, Germany). Briefly, sections were dewaxed, rehydrated, and digested with 20 μg/mL proteinase K in 10 mmol/L Tris-HCl buffer for 20 minutes and then treated with permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 minutes on ice. TUNEL reaction mixture (50 μL) was added to each sample for 30-minute incubation at 37°C. Slides were rinsed with PBS and analyzed by fluorescence microscopy. The mean numbers of apoptotic cells were determined by randomly counting five ×20 fields for each sample.

Statistical Analysis

The results are expressed as mean ± SD. Levels of significance were evaluated by a two-tailed, paired Student's *t* test or *F* test (ANOVA), and $P < 0.05$ was considered significant. We did a two-way ANOVA to test for differences among treatments and among cell lines. The model included a term for the interaction between treatment and cell line. We used Tukey's multiple comparisons procedure to determine how the treatments and cell lines are different if the test from the ANOVA for these effects were significant. This procedure controls the experiment-wise type I error rate (false-positive rate) at 0.05.

For assessment of the *in vivo* xenograft studies, we found the percent change in mean tumor size from baseline (day 1) to each subsequent day when tumor size was measured. A comparative analysis of the percent change in the mean tumor size was fit with a regression model that included terms for treatment, day, and treatment × day interaction while forcing the intercept term to be 0. A *t* test was used to assess whether each term in the model was statistically significant at the 0.05 level. Data are summarized as mean ± SD by treatment group or study day as appropriate.

Results

HER2-Overexpressing Breast Cancer Cells Are More Sensitive to Growth-Inhibitory Effects of CDDO

Overexpression of HER2 in transfected MCF7 and MDA-MB-435 cells was confirmed by Western blot analysis (Fig. 1A). Transfection did not significantly change ER status, with MCF7 lines remaining ER positive and MDA-MB-435 cells being ER negative. HER2-transfected MCF7 cells and their vector (control) counterparts were treated with 1 to 5 μmol/L CDDO. HER2-overexpressing cells were more sensitive to growth-inhibitory effects of CDDO as determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay and IC_{50} analyses (Fig. 1B); these differences were statistically significant as determined by ANOVA

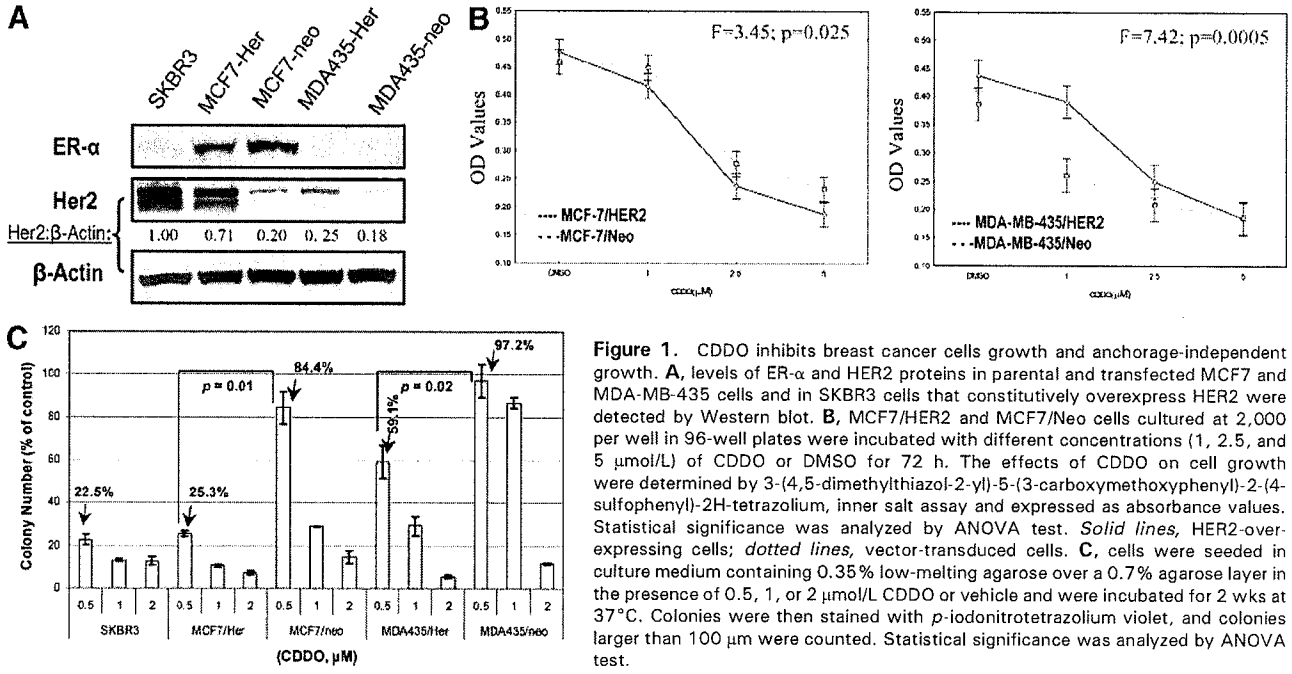


Figure 1. CDDO inhibits breast cancer cells growth and anchorage-independent growth. **A,** levels of ER-α and HER2 proteins in parental and transfected MCF7 and MDA-MB-435 cells and in SKBR3 cells that constitutively overexpress HER2 were detected by Western blot. **B,** MCF7/HER2 and MCF7/Neo cells cultured at 2,000 per well in 96-well plates were incubated with different concentrations (1, 2.5, and 5 μmol/L) of CDDO or DMSO for 72 h. The effects of CDDO on cell growth were determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay and expressed as absorbance values. Statistical significance was analyzed by ANOVA test. *Solid lines,* HER2-overexpressing cells; *dotted lines,* vector-transduced cells. **C,** cells were seeded in culture medium containing 0.35% low-melting agarose over a 0.7% agarose layer in the presence of 0.5, 1, or 2 μmol/L CDDO or vehicle and were incubated for 2 wks at 37°C. Colonies were then stained with *p*-iodonitrotetrazolium violet, and colonies larger than 100 μm were counted. Statistical significance was analyzed by ANOVA test.

(P = 0.025 and P = 0.0005, respectively, in MCF7 and MDA-MB-435 cells). The IC_{50}s were 3.53 ± 0.17 and 4.03 ± 0.12 μmol/L, respectively, in MCF7/HER2 and MCF7/Neo cells (P = 0.002); 3.86 ± 0.15 and 7.47 ± 1.04 μmol/L, respectively, in MDA-MB-435/HER2 and MDA-MB-435/Neo cells (P = 0.018).

To determine whether CDDO affects anchorage-independent cell growth, an important hallmark of cell transformation, we monitored colony formation in soft agarose. A two-way ANOVA was used to compare the differences among different concentrations of CDDO (0.5, 1.0, and 2.0 μmol/L) and among cell lines (MCF7/HER, MCF7/Neo, MDA-MB-435/HER, and MDA-MB-435/Neo). This analysis revealed that there are differences among the treatments (P < 0.001), and there are differences among the cell lines (P < 0.001). When a term for the interaction between cell line and treatment was included in the ANOVA model, this term was also statistically significant (P < 0.001), suggesting that the treatments have different effects in different cell lines. Tukey's multiple comparisons test revealed that all three treatments are different from one another (P = 0.002), with means of 66.514 (CDDO 0.5 μmol/L), 38.787 (CDDO 1.0 μmol/L), and 9.870 (CDDO 2.0 μmol/L). Tukey's multiple comparisons procedure also revealed that all four cell lines are significantly different from one another (P < 0.001), with means of 65.196 (MDA-MB-435/Neo), 31.370 (MDA-MB-435/HER), 42.579 (MCF7/Neo), and 14.416 (MCF7/HER). This analysis shows that the colony-forming activity of HER2-overexpressing breast cancer cells is significantly more suppressed than that of nonoverexpressing cell lines. The size of colony was profoundly diminished in HER2-overexpressing MCF7 and MDA-MB-435 cells compared with their vectors (control) counterparts (data not shown). At ≥2 μmol/L CDDO, colony formation was almost completely suppressed in all the cell lines tested. This finding implies that at low concentrations of CDDO HER2 is likely to be the primary target for the reduction of colony formation. At higher concentrations of CDDO, other mechanisms may apply, such as cell cycle regulation and/or an induction of apoptosis.

CDDO Inhibits HER2 Phosphorylation and Decreases HER2 Kinase Activity and Protein Level

To determine if preferential inhibition of HER2-overexpressing cells by CDDO is associated with potential effects of the compound on HER2 signaling, we examined the effects of CDDO on HER2 phosphorylation and tyrosine kinase activity. CDDO treatment (1 hour) inhibited phosphotyrosine content of HER2 in MCF7/HER2 cells along with specific phosphorylation of the 1248 site of HER2 (Fig. 2A).

An immunocomplex kinase assay was then done to examine the effects of CDDO on the tyrosine kinase activity of HER2. Immunoprecipitates of lysates of MCF7/HER2 cells were incubated in the presence of varying concentrations of CDDO or vehicle. Both the autophosphorylation of HER2 and the transphosphorylation of an exogenous substrate, enolase, were partially inhibited by CDDO (Fig. 2B). Time-course analysis further showed that CDDO decreased the levels of total HER2 protein in MCF7/HER2 cells at 24 and 48 hours in a dose-dependent fashion, but this effect was apparent only at high (>5 μmol/L) concentrations of CDDO (Fig. 2C). No effect on HER2 mRNA was seen at 24 hours as shown by Northern blot analysis (Fig. 2D). These results suggest that CDDO represses the intrinsic tyrosine kinase activity of HER2

and subsequently reduces total HER2 protein levels. To investigate the possibility that CDDO induces the proteasomal degradation of HER2, cells were pretreated with the proteasome inhibitors MG132 or proteasome inhibitor I and then exposed to CDDO. Both proteasome inhibitors prevented a decrease in the level of HER2 protein (Fig. 2E), suggesting that CDDO indeed induces the proteasomal degradation of the receptor. However, proteasome inhibitors failed to restore cyclin D1 levels.

PPARγ-Dependent Transactivation by CDDO in Breast Cancer Cells

To determine if the putative target of CDDO, PPARγ, is modulated by CDDO in HER2-overexpressing cells, we transfected MCF7/Neo and MCF7/HER2 cells with a PPRE-Luc construct and measured the luciferase activity. PPARγ protein was expressed in both MCF7/Neo and MCF7/HER2 cells although at higher levels in the MCF7/HER2 cells (Fig. 3A). We compared transcriptional activation of the receptor in both cell lines by CDDO and PPARγ ligand ciglitazone (Fig. 3B). A two-way ANOVA that included a term for the interaction between cell line and treatment was done to test for differences among treatments (CDDO 2.0 μmol/L, ciglitazone 10.0 μmol/L, and DMSO) and between cell lines (MCF7/HER and MCF7/Neo). This analysis revealed that there are differences among the treatments ($P < 0.001$), and the treatments have different effects in different cell lines ($P < 0.001$). A one-way ANOVA was then used to compare the transcriptional activation of PPARγ by CDDO or ciglitazone within each cell line. For both cell lines, there was a statistically

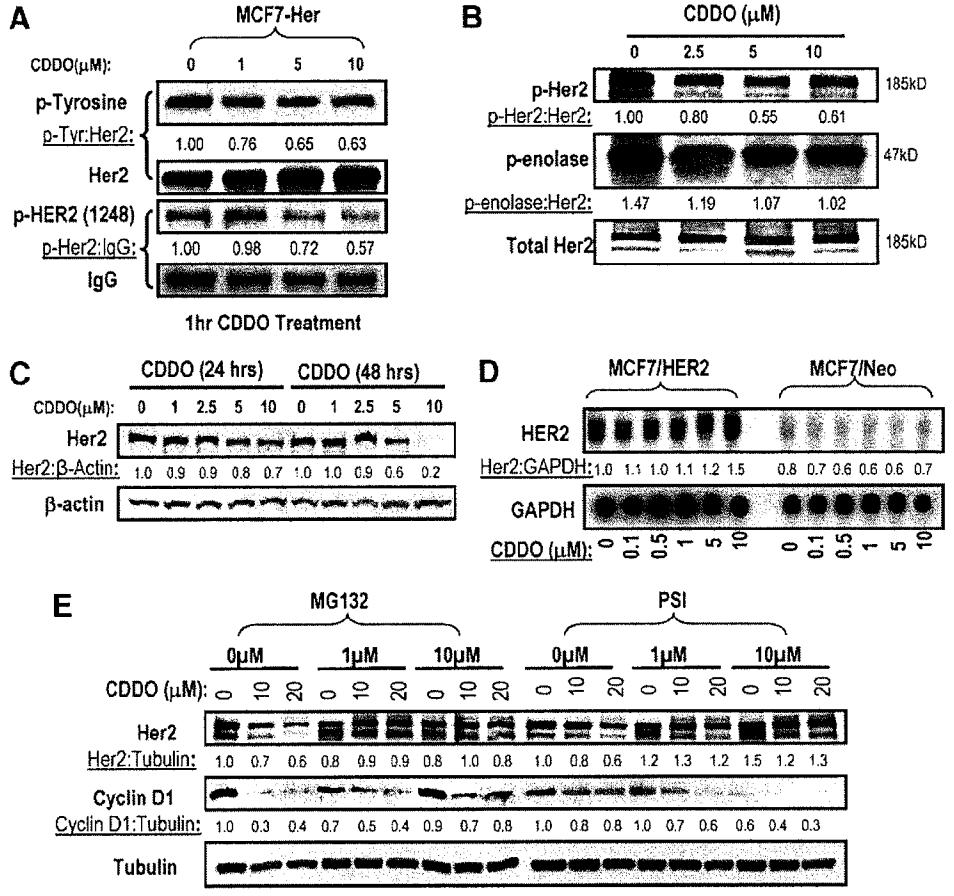


Figure 2. CDDO decreases HER2 activation and protein level. **A,** MCF7/HER2 cells were incubated with DMSO or increasing concentrations of CDDO (1, 5, and 10 μmol/L) for 1 h. Cell extracts were immunoprecipitated with the anti-HER2 antibody and then immunoblotted with either anti-phosphotyrosine antibody (*p-Tyrosine*), phosphospecific HER2 antibody [*p-HER (1248)*], or anti-HER2 antibody. IgG as a loading control. Densitometric analysis was done as described in Materials and Methods. *Numbers,* ratios of phospho-HER2 to total HER2. **B,** cell lysates from untreated MCF7/HER2 cells were immunoprecipitated with the anti-HER2 antibody. Kinase activity was measured by incubation with [γ-^{32}P]ATP, enolase, and varying concentrations of CDDO for 20 min. Reactants were resolved on 10% SDS-PAGE and transferred to nitrocellulose, and the phosphorylation products were visualized by autoradiography. Total HER2 was detected by Western blot as a loading control. **C,** MCF7/HER2 cells were exposed to the indicated concentrations of CDDO or DMSO for 24 or 48 h and then harvested for Western blot analysis with anti-HER2 antibody. **D,** Northern blot in which HER2 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probes were used for detecting mRNA levels of MCF7/HER2 and MCF7/Neo cells treated with the indicated concentrations of CDDO for 24 h. **E,** CDDO-induced HER2 down-regulation is partially inhibited by proteasome inhibitors. MCF7/HER2 cells were pretreated for 1 h with the indicated concentrations of the proteasome inhibitors MG132 or proteasome inhibitor I followed by exposure to CDDO for 16 h, and cell lysates were analyzed by Western blot.

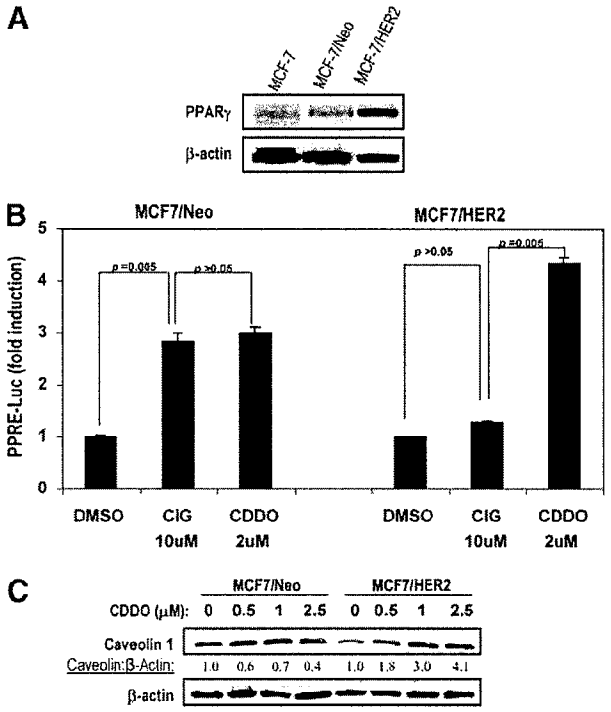


Figure 3. **A**, expression of PPARγ in parental MCF7 cells, MCF7/Neo vector control cells, and MCF7/HER2 cells as shown by Western blot analysis using a monoclonal antibody to PPARγ. The monoclonal antibody reacts with both PPARγ1 and PPARγ2 isoforms. **B**, MCF7/Neo and MCF7/HER2 cells were transiently transfected with 1 μg TK-PPRE-Luc construct. After 24 h, transfected cells were treated with 2 μmol/L CDDO or 10 μmol/L ciglitazone (*CIG*), and luciferase activity was determined as described in Materials and Methods. *Columns*, mean (n = 3); *bars*, SD. **C**, MCF7/Neo and MCF7/HER2 cells were treated with indicated concentrations of CDDO for 24 h, and caveolin-1 expression was determined by Western blot analysis.

significant difference among the treatment groups (P = 0.002 and P < 0.001 for MCF7/Neo and MCF7/HER2, respectively). In MCF7/Neo cells, comparison by Tukey's multiple comparisons procedure revealed that 2.0 μmol/L CDDO and 10.0 μmol/L ciglitazone were not significantly different from one another but that both of these treatments were significantly different from DMSO (P = 0.005). In MCF7/HER2, 2.0 μmol/L CDDO was significantly different from both 10.0 μmol/L ciglitazone and DMSO (P = 0.005) but that 10.0 μmol/L ciglitazone and DMSO were not significantly different from one another. These data suggest that CDDO activates PPARγ signaling in both HER2-positive and HER2-negative cells. In contrast, the PPARγ ligand ciglitazone at 10 μmol/L induced increased luciferase activity in MCF7 (parental) and MCF7/Neo cells but not in MCF7/HER2 cells, consistent with previously published results (28).

Caveolin-1 is a potent suppressor of mammary tumor growth and metastasis (29), and recent studies have shown that caveolins are induced in cancer cells when PPARγ is ligated (30–32). Because caveolin-1 negatively regulates the activation of diverse kinases, including HER2 (33), and the activation of PPARγ has been reported to inhibit neuregulin-induced HER2 tyrosine phosphorylation (17), we examined the effects of CDDO on caveolin-1 expression in breast cancer cells. MCF7/HER2 cells expressed caveolin-1 at significantly lower levels than MCF7/Neo controls, consistent with published reports (ref. 33; Fig. 3C). CDDO consistently induced caveolin-1 protein expression in both cell lines at 24 hours, restoring caveolin-1 expression in MCF7/HER2 cells to the levels in vector-transduced cells (Fig. 3C).

We showed previously that CDDO down-regulates cyclin D1 in breast cancer cells (24). In this study, we have now extended our analysis to cells overexpressing HER2. By using quantitative real-time PCR, we were able to determine that CDDO down-regulated cyclin D1 mRNA at 24 hours in a dose-dependent fashion in both MCF7/Neo and MCF7/HER2 cells (Fig. 4A), with the complete disappearance of cyclin D1 protein at 5 and 10 μmol/L CDDO (Fig. 4B). However, this effect was not seen at lower (1 and 2.5 μmol/L) concentrations of CDDO (data not shown). No change in cyclin E expression was noted.

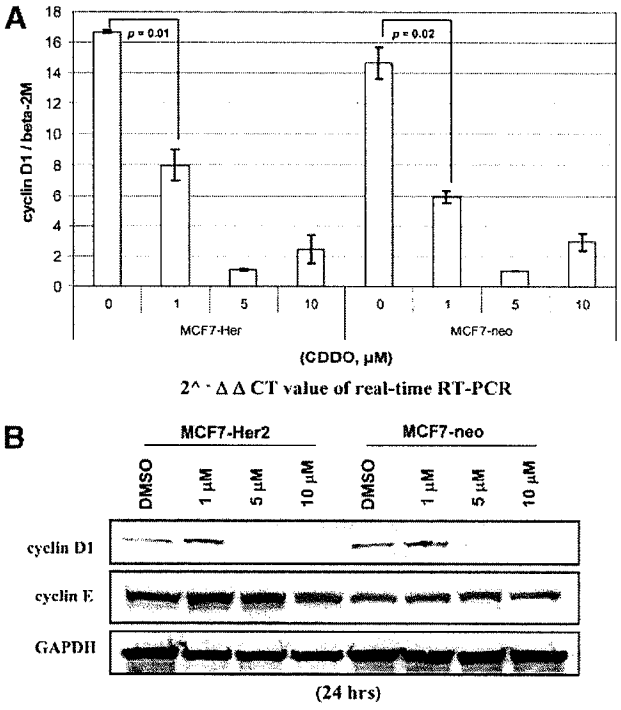
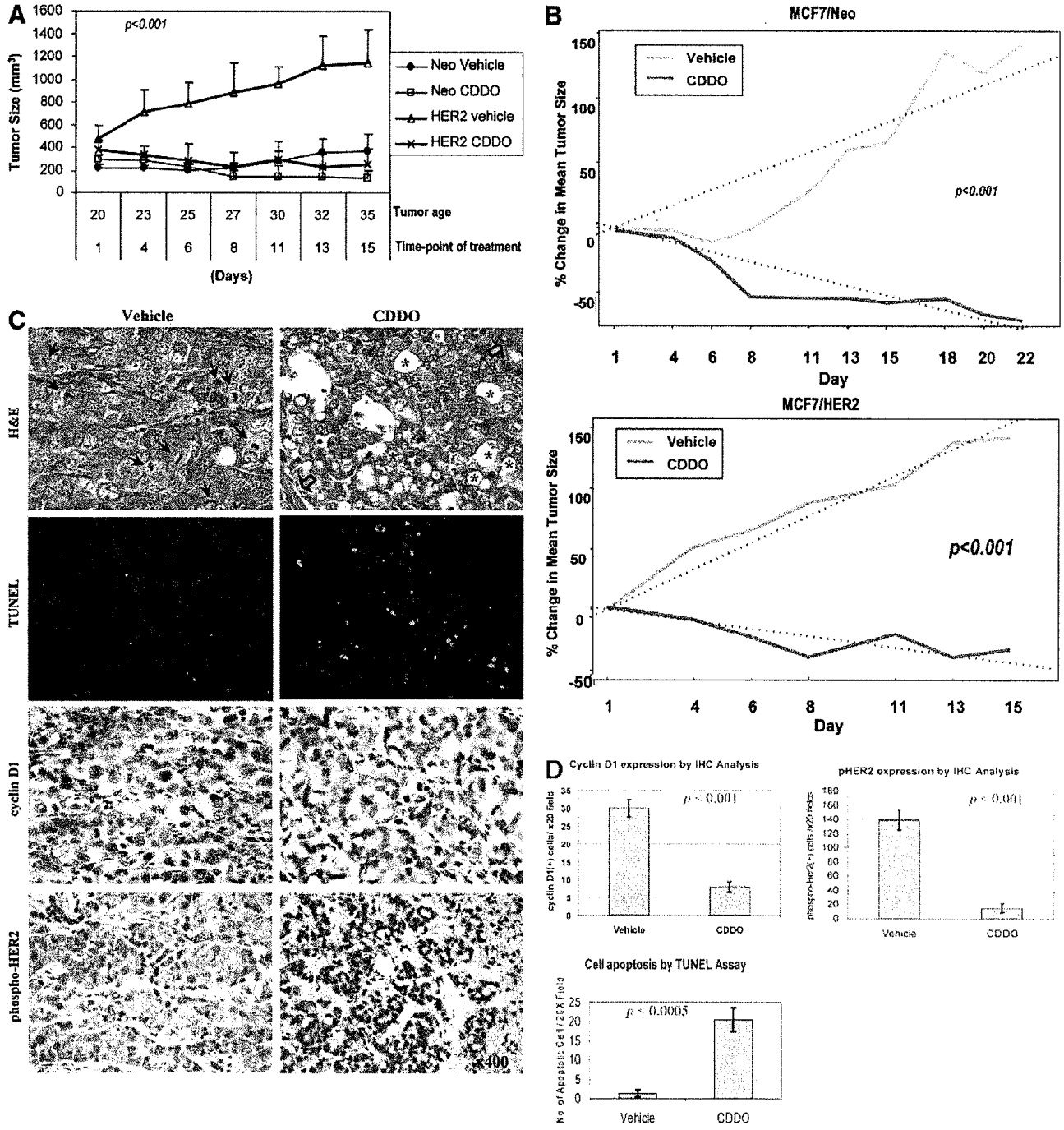


Figure 4. CDDO induces cyclin D1 down-regulation in MCF7/Neo and MCF7/HER2 cells. **A**, breast cancer cells were exposed to the indicated concentrations of CDDO or vehicle for 24 h. RNA extraction and real-time PCR were done to determine the effects of each concentration of CDDO. Each sample was normalized to β_2-microglobulin, and results were expressed as a fold change. **B**, in the same experiment, cells were harvested and lysed for Western blot analysis. Western blot analysis confirmed the results of real-time PCR at the protein level, with the down-regulation of cyclin D1 and no change in cyclin E expression.

CDDO Reduces HER2-Positive Breast Cancer Growth in Immunodeficient Mice

We then tested the antitumor effects of CDDO *in vivo* in a murine breast cancer xenograft model. At the start of therapy, MCF7/HER2 tumors were twice as large as MCF7/Neo tumors and continued to grow faster. However, CDDO abrogated the growth of both tumor types compared with the tumors in control mice treated with vehicle alone (Fig. 5A). Indeed, there was a statistically significant difference ($P < 0.001$) in the rate of tumor growth and the percentage of change in the mean tumor sizes over time between the two treatment groups (vehicle and CDDO), with the same conclusion reached in mice with either MCF7/Neo or MCF7/HER2 tumors (Fig. 5B).

We then analyzed the histologic appearance of tumors collected from the mice following treatment. Microscopically,



changes secondary to CDDO treatment include inconspicuous mitotic figures and reduced nucleocytoplasmic ratio, tumor tissue replacement by distinct fibrosis that trend to confluence and separate the tumor mass into multifocal nest structures, formation of ductal lumen-like structure (Fig. 5C, H&E), accumulation of foamy cytoplasm in tumor cells, and inflammatory cell infiltration (data not shown). Furthermore, CDDO significantly ($P < 0.001$) decreased HER2 phosphorylation and nuclear cyclin D1 expression in tumors as determined by immunohistochemistry analysis (Fig. 5C and D). TUNEL assay showed that CDDO treatment induced tumor cell apoptosis (Fig. 5C, TUNEL), which was 7-fold higher in CDDO-treated compared with vehicle-treated group (Fig. 5C and D; $P < 0.0005$).

Discussion

In this study in which we examined the antitumor effect of the triterpenoid CDDO on human breast cancer cells engineered to overexpress the HER2/*neu* gene, CDDO inhibited the growth of cells overexpressing HER2 at concentrations lower than those required to inhibit the growth of vector control cells. We further found *in vitro* that CDDO inhibited HER2 autophosphorylation and transphosphorylation. The concentrations of CDDO required for the inhibition of kinase activity were in the same range as concentrations that inhibit breast cancer cell growth. At 0.5 and 1 μmol/L, the colony formation of HER2-overexpressing cell lines was significantly more suppressed than that of the nonoverexpressing cell lines in soft agar. At higher concentrations, the colony-forming ability was almost completely suppressed, regardless of the level of HER2 expression. At high concentrations, CDDO decreased the total HER2 levels likely by proteasome-dependent degradation. It has been reported that the degradation of HER2 by 17-allylamino,17-demethoxygeldanamycin in parental MCF7 cells expressing low HER2 levels was not sufficient to arrest cell growth but caused the death of cells that depended on HER2 signaling (34). These collective data suggest that although CDDO preferentially suppresses the growth and transformation ability of HER2-overexpressing cancer cells at lower concentrations, other effects of CDDO may account for its inhibitory effect on growth of cell lines expressing the basal level of HER2.

CDDO reportedly binds to and transactivates PPARγ (14), and we have reported that CDDO activates PPARγ in leukemic cells (23) and breast cancer cells (24). In keeping with this, human breast cancer cells express high levels of PPARγ and are functionally responsive to synthetic and natural ligands (35, 36). Moreover, PPARγ is expressed in both primary and metastatic breast cancer, where its transactivation has been reported to induce growth arrest and apoptosis (37) and to mediate the transcription of target genes that are associated with a more differentiated, less malignant phenotype (38). Recent studies have further shown that the overexpression of PPARγ in breast cancer cells is caused by a tumor-specific promoter that is distinct from the promoter used in normal epithelial cells (39). PPARγ has also been suggested to be a crucial gene for regulating BRCA1 gene expression, which might therefore make it important in the BRCA1 regulatory pathway that is involved in the pathogenesis of sporadic breast cancer (40).

A recent study showed that PPARγ levels are up-regulated in cells that overexpress HER2, but this was associated with resistance to the growth-inhibitory effects of the bona fide PPARγ ligand troglitazone unless it was combined with trastuzumab (Herceptin; ref. 28). Consistent with these findings, we also observed higher PPARγ protein levels in MCF7/HER2 cells than in MCF7/Neo cells. Furthermore, ciglitazone, another PPARγ ligand from the thiazolidinedione class, failed to activate PPARγ. In contrast, CDDO induced PPARγ-dependent transactivation in both HER2-transfected and vector control cells. This difference in tissue/cell selectivity between CDDO and thiazolidinediones in their PPARγ-dependent responses may result from the unique ability of CDDO to recruit different classes of coactivators. As such, our data in colon cancer cells showed recruitment of multiple coactivators (Src-1, Src-2, Src-3, TRAP220/DRIP205, CARM-1, and PGC-1) that is qualitatively different from that induced by other PPARγ ligands (41).

We recently identified the cell cycle regulator genes cyclin D1 and p21 as transcriptional targets of CDDO in breast cancer (24). Similar to its effects in other cell lines, in the present study, we observed that CDDO down-regulated cyclin D1 expression at both mRNA and protein levels in MCF7/HER2 cells. Cyclin D1 is a major regulator of G_1

Figure 5. Effect of CDDO on breast cancer *in vivo*. **A,** female nude mice were inoculated s.c. with estrogen-releasing pellets and with 5×10^6 MCF7/HER2 or MCF7/Neo cells. Twenty days after inoculation, five MCF7/Neo-injected mice and eight MCF7/HER2-injected mice were treated with liposomally encapsulated CDDO (20 mg/kg/d i.v. thrice weekly for 3 wks). Eleven mice (4 injected with MCF7/Neo and 7 injected with MCF7/HER2 cells) received vehicle only. The tumor was measured twice weekly with microcalipers, and the tumor volume was calculated. **B,** percent change in the mean tumor size over time in the mice injected with MCF7/Neo cells (*top*) and MCF7/HER2 cells (*bottom*). *Green lines,* percent change in the mean tumor size each day in the mice treated with vehicle; *red lines,* percent change in the mean tumor size each day in the mice treated with CDDO; *dotted line,* regression line obtained when the percent change in the mean tumor size is fit with a simple linear model over days, with the intercept term forced to be 0. **C,** tumors from mice that received vehicle control or CDDO were excised and stained with H&E, TUNEL, or antibodies against cyclin D1 and phospho-HER2. Microscopically, tumor cells from vehicle-treated animals appeared larger with high nucleocytoplasmic ratio and increased mitotic figures (*closed arrowheads*). Tumor cells from mice treated with CDDO showed reduced nucleocytoplasmic ratio and mitotic figures. Tumor tissue is replaced by distinct loose fibrosis (*open arrows*) and formed ductal lumen-like structure (*asterisks*), and the number of apoptotic cells is increased (*green signal* in TUNEL staining). Immunohistochemically, positive signal (*brown staining*) of cyclin D1 was mostly of nuclear localization and positive signal (*brown staining*) of phospho-HER2 was located on cell membrane. Tumor cells after CDDO treatment showed weak or lack of positive signals of cyclin D1 and phospho-HER2. **D,** cyclin D1 and phospho-HER2 expression (*IHC*) and cell apoptosis (TUNEL) were analyzed as described in Materials and Methods. *Columns,* mean number of positive cells was counted in three to five random areas of ×20 field for each sample; *bars,* SD. Results were compared by *t* test.

cell cycle progression, with up to 40% of human breast cancers showing overexpression or amplification of cyclin D1 (42, 43). Cyclin D1 in the mammary gland is required for the development of HER2/*neu*- or Ras-induced breast cancers (44). Conversely, cyclin D1 antisense abolished the growth of Neu-transformed mammary cells in immunodeficient mice (45). Further, Wang et al. (46) reported that 15-deoxy-Δ12,14-prostaglandin J2 decreased cyclin D1 mRNA and protein levels in MCF7 cells, and their studies indicated that this transcriptional inhibition was due to competition between PPARγ and c-fos (bound to the cyclin D1 promoter) for limiting cellular levels of p300, an important coregulatory protein. In another study, 15-deoxy-Δ12,14-prostaglandin J2 and ciglitazone decreased cyclin D1 protein levels by the proteasome-dependent degradation of cyclin D1 (47). In the present study, CDDO caused the down-regulation of cyclin D1 protein at concentrations higher than those required to inhibit cell growth, indicating that this response was PPARγ independent as was also reported for 15-deoxy-Δ12,14-prostaglandin J2 and substituted diindolylmethane derivatives in MCF7 cells (21, 48, 49). However, unlike other PPARγ ligands (47), CDDO did not induce the degradation of cyclin D1 via the ubiquitin-proteasome pathway as shown by the fact that the proteasome inhibitors MG132 and proteasome inhibitor I failed to restore cyclin D1 expression. These observations clearly distinguish between the PPARγ-independent responses induced by CDDO and by other structural classes of PPARγ agonists.

Caveolin-1 recently emerged as a potential downstream target of the PPARγ gene in several cellular systems. Caveolin-1, the principal structural component of the caveolae membrane domains in mammary epithelial cells, is a potent suppressor of mammary tumor growth and metastasis (29, 50). Caveolins may function as negative regulators of signal transduction through their direct interactions with caveolae-associated signaling molecules, such as Ha-Ras, epidermal growth factor receptor, protein kinase C, Src family kinases, and others (51–53), and recent studies have shown a reciprocal relationship between the HER2 tyrosine kinase activity and caveolin-1 protein expression (33). Further, caveolin-1 transcriptionally controls cyclin D1 levels in different cell types (54). Because several studies (30–32) have shown that caveolins are modulated via PPARγ ligation, we examined the effects of CDDO on caveolin-1 expression in breast cancer cells. CDDO significantly induced caveolin-1 expression in both MCF7/Neo and MCF7/HER2 cells in only 24 hours and thereby restored caveolin-1 expression in HER2 transfectants with low baseline levels. Because caveolin-1 exhibits tumor-suppressing and growth-inhibitory activities, these results suggest that the induction of caveolin-1 is important for the antiproliferative effects of PPARγ-active CDDO in breast cancer. We have recently reported that CDDO-induced growth inhibition of colon cancer cell correlated with induction of caveolin-1 in PPARγ-dependent fashion (41). Further studies will need to be done to determine whether the induction of caveolin-1 by CDDO is responsible for the inhibition of HER2 tyrosine activity and cyclin D1 down-regulation. Of relevance, the natural PPARγ ligand 15-deoxy-Δ12,14-prostaglandin J2 was shown to block the phosphorylation of HER2 and other receptor tyrosine kinases, such as insulin-like growth factor-1 receptor (17). However, our results show that CDDO directly inhibits HER2-dependent phosphorylation activity *in vitro* (Fig. 5B), suggesting that this inhibitory response is also PPARγ independent.

Several studies have shown the chemopreventive activity of PPARγ ligands in chemically induced mammary tumors, an activity that was enhanced by retinoid X receptor ligation (55–57). Our study provides the first *in vivo* evidence of the antitumor activity of liposomally delivered CDDO in a xenograft model of breast cancer. CDDO completely abrogated the growth of both MCF7/Neo and MCF7/HER2 tumors in an immunodeficient mouse model. This is particularly notable because the MCF7/HER2 cells otherwise grew very rapidly, requiring the sacrifice of animals in the control group by day 35. Importantly, we also showed that CDDO down-regulates cyclin D1 and phospho-HER2 expression and induces tumor cell apoptosis *in vivo*, which was consistent with our *in vitro* data. Morphologically, tumors exhibited reduced nucleocytoplasmic ratio and lower mitotic index, consistent with the low cyclin D1 expression level. Tumor cells also contained clear vacuolated or foamy cytoplasm after CDDO treatment. These results suggest that CDDO may be beneficial for treatment of patients with breast cancer involving HER2 amplification, which is historically an aggressive disease associated with short survival. Further recommending CDDO for clinical use, CDDO showed a favorable toxicity and pharmacokinetic profile in preclinical studies conducted at the National Cancer Institute (58), and clinical phase I trials of CDDO in patients with hematopoietic malignancies and solid tumors have been planned.

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Cell, Tumor, and Stem Cell Biology

The Novel Triterpenoid C-28 Methyl Ester of 2-Cyano-3, 12-Dioxoolen-1, 9-Dien-28-Oic Acid Inhibits Metastatic Murine Breast Tumor Growth through Inactivation of STAT3 Signaling

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We and others have reported that C-28 methyl ester of 2-cyano-3, 12-dioxoolen-1, 9-dien-28-oic acid (CDDO-Me) effectively inhibits the growth of multiple cancer cell types. Our previous studies indicated that prolonged CDDO-Me treatment inactivated extracellular signal-regulated kinase signaling in acute myelogenous leukemia cells. Whether treatment with CDDO-Me has an earlier effect on other proteins that are important for either signal transduction or oncogenesis is unknown. Constitutively activated signal transducer and activator of transcription 3 (STAT3) is frequently found in human breast cancer samples. Constitutively activated STAT3 was shown to up-regulate c-Myc in several types of cancer and has a feedback effect on Src and Akt. To examine the effects of CDDO-Me on STAT3 signaling in breast cancer, we used the murine 4T1 breast tumor model, which is largely resistant to chemotherapy. *In vitro*, after treatment of 4T1 cells with 500 nmol/L CDDO-Me for 2 h, we found (*a*) inactivation of STAT3, (*b*) inactivation of Src and Akt, (*c*) 4-fold reduction of c-Myc mRNA levels, (*d*) accumulation

of cells in G_2-M cell cycle phase, (*e*) abrogation of invasive growth of 4T1 cells, and (*f*) lack of apoptosis induction. In *in vivo* studies, CDDO-Me completely eliminated 4T1 breast cancer growth and lung metastases induced by 4T1 cells in mice when treatment started 1 day after tumor implantation and significantly inhibited tumor growth when started after 5 days. *In vivo* studies also indicated that splenic mature dendritic cells were restored after CDDO-Me treatment. In summary, these data suggest that CDDO-Me may have therapeutic potential in breast cancer therapy, in part, through inactivation of STAT3. [Cancer Res 2007;67(9):4210–17]

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Interim results of a phase I trial with a novel orally administered synthetic triterpenoid RTA 402 (CDDO-Me) in patients with solid tumors and lymphoid malignancies.

Sub-category: Antiangiogenic or Antimetastatic Agents
Category: Developmental Therapeutics: Molecular Therapeutics
Meeting: 2007 ASCO Annual Meeting

Abstract No: 14101

Citation: *Journal of Clinical Oncology*, 2007 ASCO Annual Meeting Proceedings Part I. Vol 25, No. 18S (June 20 Supplement), 2007: 14101

Author(s): B. J. Dezube, R. Kurzrock, J. P. Eder, J. G. Supko, C. J. Meyer, L. H. Camacho, M. Andreeff, M. Konopleva, L. Lescale-Matys, D. Hong

Abstract:

Background: RTA 402 (CDDO-Me) is a novel synthetic triterpenoid with potent anticancer and anti-inflammatory activity through selective modulation of proteins that respond to changes in oxidative stress, including NF-?B, JNK, and STAT3. Based on preclinical data demonstrating effects on tumors and associated stroma, a Phase 1 dose-finding and pharmacokinetic study was initiated.

Methods: RTA 402 is administered orally once a day for the first 21 days of a 28-day cycle. Dose escalation is proceeding according to an accelerated titration design until an MTD is reached.

Results: RTA 402 has been administered to 11 patients at 7 dose levels (5 to 300 mg/day). Cycle 1 data are available for 8 patients. No significant drug-related toxicity has been reported and dose escalation continues in 100% increments. The median biological half-life of RTA 402 was 49 h (range, 18-67 h), and all patients receiving doses >20 mg/day were continuously exposed to plasma levels of the drug exceeding 1 ng/mL. Evidence of antitumor and biological activity has been observed in initial patients. A patient with medullary thyroid carcinoma treated at 5 mg/day has received eight cycles of therapy and has had stable disease with a 70% reduction of calcitonin levels. A patient with metastatic melanoma has experienced disease stabilization through four cycles and remains on study. A second melanoma patient has also experienced disease stabilization through two cycles and remains on study. Radiographic evidence of regressing lesions has been observed in both melanoma patients. Additionally, significant reductions in circulating VEGF, MMP-9, TNF, IL-8, and IL-10, up to 65%, 91%, 90%, 73%, and 70%, respectively, have been observed in at least half of patient samples. Cytokine suppression has correlated with improvements in reported pain and fatigue.

Conclusions: Orally administered RTA 402 appears well tolerated up to 150 mg/day and with prolonged exposure up to 8 months. Data from initial patients indicate biological activity and suggest clinical benefit, with disease stabilization in several patients who had rapidly progressing disease with high tumor burden. Phase 2 trials are planned.

Other Abstracts in this Sub-Category

1. **Phase 1b study defining the optimal dosing combinations of the mTOR inhibitor AP23573 and Paclitaxel (PTX).**
   Meeting: 2007 ASCO Annual Meeting Abstract No: 3509 First Author: S. Cresta
   Category: Developmental Therapeutics: Molecular Therapeutics - Antiangiogenic or Antimetastatic Agents
2. **The effects of paclitaxel (PTX) and 2-methoxyestradiol (2-ME_2) on tumor oxygenation and HIF-1α in breast cancer.**
   Meeting: 2007 ASCO Annual Meeting Abstract No: 3517 First Author: K. L. Blackwell
   Category: Developmental Therapeutics: Molecular Therapeutics - Antiangiogenic or Antimetastatic Agents
3. **Phase I and biomarker study of ABT869, a multiple receptor tyrosine kinase inhibitor, in patients with refractory solid malignancies.**
   Meeting: 2007 ASCO Annual Meeting Abstract No: 3519 First Author: C. I. Wong
   Category: Developmental Therapeutics: Molecular Therapeutics - Antiangiogenic or Antimetastatic Agents

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Abstracts by B. J. Dezube

1. **Interim results of a phase I trial with a novel orally administered synthetic triterpenoid RTA 402 (CDDO-Me) in patients with solid tumors and lymphoid malignancies.**
   Meeting: 2007 ASCO Annual Meeting Abstract No: 14101 First Author: B. J. Dezube
   Category: Developmental Therapeutics: Molecular Therapeutics - Antiangiogenic or Antimetastatic Agents

4458

PPARγ NUCLEAR RECEPTOR AS A NOVEL THERAPEUTIC TARGET IN AML.

Marina Y. Konopleva, Elena Elstner, Teresa McQueen, Twee Tsao, Zeev Estrov, H. Phillip Koeffler, Michael Sporn, Michael Andreeff, University of Texas M. D. Anderson Cancer Center, Houston, TX; Charite, School of Medicine, Humboldt University, Berlin, Germany; Cedars-Sinai Medical Center, Los Angeles, CA; Dartmouth Medical School, Hanover, NH.

The peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor family that forms a heterodimer with RXR to bind DNA and activate transcription. We have demonstrated that PPARγ protein is expressed in both, myeloid and lymphoid leukemic cell lines, in primary AML, ALL and CLL samples. We tested the effect of PPARγ ligands troglitazone (TGZ), roziglitazone (BRL49653), 15dPGJ2, L-805645 (Merck), GW347845X (Glaxo) on proliferation, apoptosis and differentiation of leukemic cell lines. BRL49653, TGZ and 15dPGJ2 decreased proliferation of leukemic cell lines as determined by cell count and 3H incorporation, with 15dPGJ2 being the most potent (IC50=5-10μM). 6-day treatment with BRL49653, 15dPGJ2 and GW347845X induced CD11b expression in HL-60 cells. Combination of TGZ with ATRA in U937 and THP1 cells, or 15dPGJ2 with ATRA in HL-60 induced marked myelomonocytic differentiation followed by apoptosis of differentiated cells. TGZ+ATRA synergistically reduced colony-forming ability of THP1 and U937 cells and induced phagocytic activity in these cells. We also investigated the effect of novel synthetic triterpenoid CDDO (2-cyano-3,12-dioxoolen-1,9-dien-28-oic acid) that is able to bind and transactivate PPARγ on leukemic cell growth. Our studies demonstrate that CDDO exerts strong antiproliferative and apoptotic effects on leukemic cell lines (IC50 1μM) and primary AML and CLL samples in vitro (IC50 2μM) and decreased Bcl-2 expression in leukemic blasts. CDDO increased ara-C cytotoxicity and induced monocytic differentiation in AML. Combination of CDDO with RXR-specific ligand or with ATRA markedly decreased cell viability in myeloid leukemic cells. CDDO-mediated reduction in colony formation is more pronounced in AML progenitors compared with normal CD34+ cells. These results suggest that the novel PPARγ ligands, alone and in combination with retinoids and chemotherapy, have promise as novel therapy for leukemias. Ligation of PPARγ in combination with retinoids provides a mechanistic basis for maximal increase of transcriptional activity in target genes that control apoptosis and differentiation.

American Society of Hematology 43rd Annual Meeting and Exposition, December 7 - 11, 2001, Orlando, FL

[498] Triterpenoids CDDO and CDDO-Me Down-Regulate FLIP Expression and Sensitize AML Cells to TRAIL-Induced Apoptosis.

Won-Suk Suh, Kitada Shinichi, Youngsoo Kim, Michael Andreeff, Michael Sporn, Nanjoo Suh, John C. Reed. The Burnham Institute, La Jolla, CA, USA; MD Anderson Cancer Center, Houston, TX, USA; Dartmouth Medical School, Hanover, NH, USA

Saturday, December 8, 2001, 9:45 AM, Poster Session: Acute Leukemia - Preclinical (9:45 AM-7:30 PM) Hall C, Poster Board Number 498

Though often exhibiting initial responses to chemotherapy, Acute Myelogenous Leukemia (AML) remains a deadly disease for most adult patients, due primarily to the emergence of chemoresistant cells. Defects in apoptosis pathways make important contributions to chemoresistance, suggesting a need to restore apoptosis sensitivity in AML or to identify alternative pathways for apoptosis induction. Triterpenoids represent a class of naturally occurring and synthetic compounds with demonstrated anti-tumor activity. Some of these agents modulate PPARg activity, including CDDO (2-Cyano-3,12-Dioxoolean-1,9-Dien-28-Oic Acid) and its methyl ester (CDDO-Me), which function as weak agonists and antagonists of PPARg, respectively. Because PPARg has been linked to regulation of apoptosis-relevant genes, we explored the effects of the triterpenoid compounds CDDO and CDDO-Me on established AML cell lines (HL-60; U937; AML-2) and on freshly isolated AML blasts with respect to apoptosis and expression of apoptosis-regulatory genes. When used individually, CDDO and CDDO-Me reduced the viability of all AML lines tested in a dose-dependent manner, with effective doses for killing 50% of cells (ED50) in 48 hrs of ~ 1 uM and 0.5 uM, respectively. This loss of cell viability was attributed to apoptosis, based characteristic cell morphology and on evidence of caspase activation. Immunoblot analysis demonstrated evidence of activation of caspases-3, 7, and 8, but not 9, suggesting involvement of the "extrinsic" pathway, which has been linked to apoptosis induction by TNF-family death receptors. Accordingly, CDDO and CDDO-Me induced rapid reductions in the levels of FLIP protein, an endogenous antagonist of caspase-8 activation, without altering the levels of several other apoptosis-relevant proteins, including FADD, DR4, DR5, Bcl-2, Bcl-XL, Mcl-1, Bax, and others. Reductions in FLIP were detectable within 3 hrs after exposure of AML cell lines to CDDO or CDDO-Me, with essentially complete loss of FLIP protein expression within 6-9 hrs. The drug-induced decline in FLIP levels was dose-dependent over the concentration range of 0.1-1 uM, with partial reductions evident at 0.1 uM and >95% reduction in FLIP proteins attained with 0.5 uM or less of these compounds. CDDO- and CDDO-Me-induced reductions in FLIP protein were not secondary to caspase activation, as determined by experiments using the broad-spectrum caspase inhibitor, zVAD-fmk. FLIP reductions also preceded caspase processing in time-course experiments, using AML cell lines treated with CDDO and CDDO-Me. When used at doses that resulted in little apoptosis (O.3 uM), CDDO and CDDO-Me down-regulated FLIP and rendered AML cell lines sensitive to TRAIL, a TNF-family death ligand. In contrast, TRAIL alone failed to induce apoptosis of AML cell lines. Similar results were obtained using freshly isolated AML blasts. In contrast, apoptosis of peripheral blood lymphocytes and normal bone marrow cells was not triggered by CDDO, CDDO-Me, TRAIL, or combinations of these agents. The findings suggest that triterpenoids warrant investigation in the treatment of AML, alone or in combination with TRAIL or other immune-based therapies.

Keywords: FLIP | TRAIL | AML cells

Supported by an unrestricted educational grant from AstraZeneca.

[2209] PPARgamma Ligand CDDO Induces Apoptosis in Leukemias Via Multiple Apoptosis Pathways.

Marina Konopleva, Helene Lapillonne, Ruey-min Lee, Rui-Yu Wang, Twee Tsao, Teresa McQueen, Michael Andreeff. Blood and Marrow Transplantation, The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA; Huntsman Cancer Institute at University of Utah, Salt Lake City, UT, USA

Sunday, December 8, 2002, 5:45 PM, Poster Session: New Agents for Acute Leukemia (5:45 PM-7:15 PM) Hall D, Poster Board # 493

The peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor family that activates transcription of target genes. We have previously demonstrated that the synthetic triterpenoid CDDO (2-cyano-3,12-dioxoolen-1,9-dien-28-oic acid), that binds and transactivates PPARγ, is a potent inducer of apoptosis in both, myeloid and lymphoid leukemic cells. We have now investigated the mechanisms of CDDO-induced apoptosis. CDDO induced early mitochondrial depolarization followed by activation of caspases-8, -9 and -3. In cells with low PPARγ levels, overexpression of anti-apoptotic Bcl-2 protected from CDDO-induced killing in HL-60/Bcl-2 cells, and inhibition of Bcl-2 via Bcl-2 antisense oligonucleotides or Bcl-2 nonpeptidic inhibitor HA14-1 restored sensitivity to CDDO cytotoxicity. To determine the criticality of caspase-8 activation, we utilized Jurkat cells with mutated caspase-8 that are completely resistant to Fas ligation by Fas agonistic antibody CH-11. These cells were effectively killed by PPARγ ligand CDDO, although to a lesser degree than Jurkat cells with functional caspase-8. In the absence of caspase-8, CDDO induced caspase-9 and caspase-3 cleavage. Similarly, CDDO induced apoptosis in caspase-9 knockout mouse embryonic fibroblasts. To examine potential direct effects of CDDO on mitochondria, we evaluated cytochrome c release by CDDO in cell-free mitochondria. Both, CDDO and PPARγ ligand Rosiglitazone induced cytochrome c release in a time-dependent fashion. The peripheral benzodiazepine receptor (PBR), along with Bcl-2, is involved in the control of the mitochondrial permeability transition complex. The combination of CDDO with PBR antagonist PK11195 (100nM), that does not induce apoptosis on its own, caused significantly increased induction of apoptosis in HL-60 cells (CDDO 1 μM, 45%; CDDO+PK11195, 82%). cDNA array analysis (Affymetrix) demonstrated that CDDO caused downregulation of the genes involved in mitochondrial control in HL-60 and in MCF-7 breast cancer cells, including Bcl-2, ATP synthase H+ transporting mitochondrial F1 complex delta subunit and PBR-associated protein 1. Immunohistochemical analysis of apoptosis-inducing factor (AIF) which has been implicated in nuclear fragmentation as result of translocation from damaged mitochondria into the nucleus, showed CDDO-induced translocation of AIF from the cytosol to the nucleus. In summary, CDDO induces apoptosis via both, extrinsic and intrinsic apoptosis pathways and is capable of initiating caspase-independent cell death as a result of direct effects on mitochondria. These results suggest that novel PPARγ ligands, in particular CDDO, have promise as novel therapy for leukemias and other malignancies with documented deficiencies of different apoptosis checkpoints.

Keywords: Apoptosis\\ Leukemia\\ Nuclear receptor

1: Leukemia. 2003 Nov;17(11):2122-9.

Synthetic triterpenoids activate a pathway for apoptosis in AML cells involving downregulation of FLIP and sensitization to TRAIL.

Suh WS, Kim YS, Schimmer AD, Kitada S, Minden M, Andreeff M, Suh N, Sporn M, Reed JC.

The Burnham Institute, La Jolla, CA 92037, USA.

Acute myelogenous leukemia (AML) remains a deadly disease for most adult patients, due primarily to the emergence of chemoresistant cells. Defects in apoptosis pathways make important contributions to chemoresistance, suggesting a need to restore apoptosis sensitivity or to identify alternative pathways for apoptosis induction. Triterpenoids represent a class of naturally occurring and synthetic compounds with demonstrated antitumor activity, including 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO) and its methyl ester (CDDO-m). We explored the effects of CDDO and CDDO-m in vitro on established AML cell lines (HL-60, U937, AML-2) and on freshly isolated AML blasts. CDDO and CDDO-m reduced the viability of all AML cell lines tested in a dose-dependent manner, with effective doses for killing 50% of cells (ED(50)) within 48 h of approximately 1 and 0.5 muM, respectively. CDDO or CDDO-m also induced substantial increases in cell death in five out of 10 samples of primary AML blasts. Cell death induced by CDDO and CDDO-m was attributed to apoptosis, based on characteristic cell morphology and evidence of caspase activation. Immunoblot analysis demonstrated proteolytic processing of caspase-3, -7, and -8, but not caspase-9, suggesting the involvement of the 'extrinsic' pathway, linked to apoptosis induction by TNF-family death receptors. Accordingly, CDDO and CDDO-m induced concentration-dependent reductions in the levels of FLIP protein, an endogenous antagonist of caspase-8, without altering the levels of several other apoptosis-relevant proteins. Reductions in FLIP were rapid, detectable within 3 h after exposure of AML cell lines to CDDO or CDDO-m. CDDO and CDDO-m also sensitized two of four leukemia lines to TRAIL, a TNF-family death ligand. The findings suggest that synthetic triterpenoids warrant further investigation in the treatment of AML, alone or in combination with TRAIL or other immune-based therapies.

PMID: 12931220 [PubMed - indexed for MEDLINE]

A Synthetic Triterpenoid, CDDO-Me, Inhibits IκBα Kinase and Enhances Apoptosis Induced by TNF and Chemotherapeutic Agents through Down-Regulation of Expression of Nuclear Factor κB–Regulated Gene Products in Human Leukemic Cells

Shishir Shishodia,[1] Gautam Sethi,[1] Marina Konopleva,[2] Michael Andreeff,[2] and Bharat B. Aggarwal[1]

Abstract The C-28 methyl ester of 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO-Me), a synthetic triterpenoid based on naturally occurring ursolic and oleanolic acids, induces apoptosis in tumor cells, induces differentiation, and inhibits inflammatory response through a poorly understood mechanism. Because the nuclear transcription factor nuclear factor κB (NF-κB) has been shown to suppress apoptosis and promote proliferation and is linked with inflammation and differentiation, we postulated that CDDO-Me modulates NF-κB activity and NF-κB-regulated gene expression. Using human leukemia cell lines and patient samples, we show that CDDO-Me potently inhibits both constitutive and inducible NF-κB activated by tumor necrosis factor (TNF), interleukin (IL)-1β, phorbol ester, okadaic acid, hydrogen peroxide, lipopolysaccharide, and cigarette smoke. CDDO-Me was more potent than CDDO and its imidazole derivative. NF-κB suppression occurred through inhibition of IκBα kinase activation, IκBα phosphorylation, IκBα degradation, p65 phosphorylation, p65 nuclear translocation, and NF-κB-mediated reporter gene transcription. This inhibition correlated with suppression of NF-κB-dependent genes involved in antiapoptosis (*IAP2, cFLIP, TRAF1, survivin*, and *bcl-2*), proliferation (*cyclin d1* and c-*myc*), and angiogenesis (*VEGF, cox-2*, and *mmp-9*). CDDO-Me also potentiated the cytotoxic effects of TNF and chemotherapeutic agents. Overall, our results suggest that CDDO-Me inhibits NF-κB through inhibition of IκBα kinase, leading to the suppression of expression of NF-κB-regulated gene products and enhancement of apoptosis induced by TNF and chemotherapeutic agents.

Hematologic malignancies including leukemia, lymphoma, and myeloma account for ~10% of cancer deaths in the United States (1). The proliferation, apoptosis, and differentiation of these cells are influenced by cell signaling pathways triggered by growth factors and cytokines. The transcription factor nuclear factor κB (NF-κB) has been shown to be constitutively active in most leukemic cells and implicated in regulating the proliferation, survival, and differentiation of these cells (2–8).

Under normal conditions, NF-κB, consisting of p50, p65, and IκBα, is localized in the cytoplasm. However, when activated, this transcription factor translocates to the nucleus. In response to an activation signal, the inhibitory IκBα subunit undergoes phosphorylation, ubiquitination, and degradation, thus exposing the nuclear localization signals on the p50-p65 heterodimer. The p65 is then phosphorylated, leading to its nuclear translocation and binding to a specific sequence in DNA, which in turn results in gene transcription (9, 10). NF-κB has been shown to regulate the expression of a number of genes of which products are involved in tumorigenesis (10–14). These include antiapoptotic genes (e.g., *ciap, survivin, traf, cflip, bfl-1, bcl-2*, and *bcl-xl*), inflammatory genes (*cox-2, mmp-9*, and *VEGF*), and genes encoding adhesion molecules, chemokines, and cell cycle regulatory genes (e.g., *cyclin d1* and c-*myc*). Thus, agents that suppress NF-κB activation have therapeutic potential for leukemia and lymphoma (2–6, 8, 15).

Triterpenoids together with their close relatives, the steroids, are members of the cyclosqualenoid family (16). Natural triterpenoids are synthesized by plants as a part of their defense mechanism for regulation of physiologic processes. They have

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Note: B.B. Aggarwal is a Ransom Horne Jr. Professor of Cancer Research. M. Andreeff holds the Paul and Mary Haas Chair in Genetics.

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been used as traditional Asian medicine for centuries. The oleanolic and ursolic acids are natural triterpenoids that have been shown to possess significant anti-inflammatory and anticarcinogenic properties (17–19). Because the biological activities of some of the natural triterpenoids are relatively weak, new analogues of these molecules have been synthesized in an attempt to identify more potent agents. 2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO), a novel synthetic triterpenoid derived from oleanolic acid, has been shown to be more potent antitumor and anti-inflammatory agent than its natural analogues (20). The C-28 methyl ester of CDDO, CDDO-Me, has been shown to decrease the viability of leukemic cell lines, including multidrug resistance 1–overexpressing cells, and it was found to be more active than CDDO (21). 1[2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im) is another CDDO analogue more potent than its parent compound, CDDO, both *in vitro* and *in vivo* (20). Synthetic triterpenoids have been shown to induce proapoptotic Bax protein, inhibit the activation of extracellular signal–regulated kinase 1/2, block Bcl-2 phosphorylation (21), and down-regulate Flice-like inhibitory protein (FLIP; ref. 22). Synthetic triterpenoids also enhance transforming growth factor β/Smad signaling (23, 24).

The antitumorigenic, antiangiogenic, and proapoptotic effects, combined with the ability to suppress the expression of cyclooxygenase 2 (COX-2), inducible nitric oxide synthase, multidrug resistance gene 1, and FLIP, suggest that CDDO-Me mediates its effects through suppression of NF-κB. But whether CDDO-Me mediates its effects through modulation of NF-κB is not understood. In the current study, we examined the effect of CDDO-Me on NF-κB activation induced by various carcinogens in a large variety of tumor cell lines and in primary blast cells from leukemia patients. The results presented here show that CDDO-Me inhibits activation of NF-κB through suppression of IκBα kinase activation in these cells. This triterpenoid also inhibited the expression of antiapoptosis, proliferative, and angiogenesis genes, all known to be regulated by NF-κB.

Materials and Methods

Materials. CDDO, CDDO-Me, and CDDO-Im were synthesized by Dr. T. Honda at Dartmouth Medical College (25) and provided by Dr. Edward Sausville (Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD) through the Rapid Access to Intervention Development Program and by Dr. Michael Sporn (Dartmouth School of Medicine, Dartmouth, NH). They were dissolved in DMSO as a 10 mmol/L stock solution and stored at −20°C. Bacteria-derived human tumor necrosis factor (TNF), purified to homogeneity with a specific activity of 5×10^7 units/mg, was kindly provided by Genentech, Inc. (South San Francisco, CA). Penicillin, streptomycin, RPMI 1640, fetal bovine serum, and Lipofectamine 2000 were obtained from Invitrogen (Grand Island, NY). FuGENE 6 was purchased from Roche (Nutley, NJ). Cisplatin and Taxol were purchased from Sigma-Aldrich Co. (St. Louis, MO). The following polyclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA): anti-p65, against the epitope corresponding to amino acids mapping within the NH_2-terminal domain of human NF-κB p65; anti-p50, against a peptide 15 amino acids long, mapping at the nuclear localization sequence region of NF-κB p50; anti-IκBα, against amino acids 297-317, mapping at the COOH terminus of IκBα/MAD-3; anti-c-Rel; anti-cyclin D1, against amino acids 1 to 295, which represents full-length cyclin D1 of human origin; anti–matrix metalloproteinase (MMP)-9; anti–polyadenosine ribose polymerase; anti–inhibitor of apoptosis protein 2 (IAP2); anti-Bcl-2; and anti–TNF receptor–associated factor 1 (TRAF1). Anti-COX-2 and anti-MMP-9 antibodies were obtained from BD Biosciences (San Diego, CA) and phosphospecific anti-IκBα (Ser32) and phospho-p65 (Ser536) antibody from Cell Signaling (Beverly, MA). Anti–IκBα kinase α (IKKα) and anti-IKKβ antibodies were kindly provided by Imgenex (San Diego, CA).

Cell lines. The cell lines used in our studies included chronic myelogenous leukemia (KBM-5), human monocytic leukemia (U937), human metastatic melanoma (A375), human lymphoblastic leukemia (Jurkat), human non–small-cell lung carcinoma (H1299), human multiple myeloma (U266 and MM1), human head and neck cancer (HN5 and SCC4), and human non-Hodgkin's lymphoma (HDMYZ, HDLM2, and L428). The non-Hodgkin's lymphoma cells were kindly provided by Dr. Anas Younes (M.D. Anderson Cancer Center, Houston, TX). All other cell lines were obtained from the American Type Culture Collection (Manassas, VA). KBM-5 cells were cultured in Iscove's modified DMEM with 15% fetal bovine serum, and all other cell lines were cultured in RPMI 1640 with 10% fetal bovine serum. All media were supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin.

NF-κB activation. To determine NF-κB activation by TNF, we examined the NF-κB-DNA binding by electrophoretic mobility shift assay essentially as previously described (26). Briefly, nuclear extracts prepared from treated cells (1×10^6/mL) were incubated with ^{32}P-end-labeled, 45-mer, double-stranded NF-κB oligonucleotide (15 μg of protein with 16 fmol of DNA) from the HIV long terminal repeat, 5′-TTGTTACAA**GGGACTTTCCGCTGGGGACTTTCCA**GGGAGGCGTGG-3′ (boldface indicates NF-κB-binding sites), for 30 minutes at 37°C, and the DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5′-TTGTTACAA**CTCACTTTCCGCTGCTCACTTTC**-CAGGGAGGCGTGG-3′, was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. For supershift assays, nuclear extracts prepared from TNF-treated cells were incubated with antibodies against either the p50 or the p65 subunit of NF-κB for 30 minutes at 37°C before the complex was analyzed by electrophoretic mobility shift assay. Preimmune serum was included as a negative control. The dried gels were visualized with a Storm820 and radioactive bands were quantitated using Imagequant software (Amersham, Piscataway, NJ).

Western blot analysis. To determine the effect of CDDO-Me on TNF-dependent IκBα phosphorylation, IκBα degradation, p65 translocation, and p65 phosphorylation, Western blotting was done as previously described (19). Briefly, cytoplasmic or nuclear extracts were prepared and fractionated by SDS-PAGE. After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, blotted with the relevant antibody, and detected by enhanced chemiluminescence reagent. The bands obtained were quantitated using NIH Image (NIH, Bethesda, MD).

Immunolocalization of NF-κB p65. The effect of CDDO-Me on TNF-induced nuclear translocation of p65 was examined by an immunocytochemical method using an epifluorescence microscope (Labophot-2; Nikon, Tokyo, Japan) and a Photometrics Coolsnap CF color camera (Nikon, Lewisville, TX) as previously described (19).

IKK assay. To determine the effect of CDDO-Me on TNF-induced IKK activation, we analyzed IKK by a method essentially as previously described (19). Briefly, the IKK complex from whole-cell extracts was precipitated with antibody against IKKα and IKKβ and then treated with protein A/G-Sepharose beads (Pierce, Rockford, IL). After 2 hours, the beads were washed with lysis buffer and then resuspended in a kinase assay mixture containing 50 mmol/L HEPES (pH 7.4), 20 mmol/L $MgCl_2$, 2 mmol/L DTT, 20 μCi [γ-^{32}P]ATP, 10 μmol/L unlabeled ATP, and 2 μg of substrate glutathione *S*-transferase-IκBα (amino acids 1-54). After incubation at 30°C for 30 minutes, the reaction was terminated by boiling with SDS sample buffer for 5 minutes. Finally,

the protein was resolved on 10% SDS-PAGE, the gel was dried, and the radioactive bands were visualized with a Storm820. To determine the total amounts of IKK-α and IKK-β in each sample, 50 μg of whole-cell proteins were resolved on 7.5% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with either anti-IKK-α or anti-IKK-β antibody.

NF-κB-dependent reporter gene transcription. The effect of CDDO-Me on TNF-induced NF-κB dependent reporter gene transcription in A293 cells was measured as previously described (19). Briefly, A293 cells (5×10^5/well) were plated in six-well plates and transiently transfected by the calcium phosphate method with pNF-κB-SEAP (0.5 μg). To examine TNF-induced reporter gene expression, we transfected the cells with 0.5 μg of the SEAP expression plasmid and 2 μg of the control plasmid pCMVFLAG1 DNA for 24 hours. We then treated the cells for 8 hours with CDDO-Me and then stimulated them with 0.1 nmol/L TNF. The cell culture medium was harvested after 24 hours of TNF treatment. Culture medium was analyzed for SEAP activity according to the protocol essentially as described by the manufacturer (Clontech, Palo Alto, CA) using a Victor 3 microplate reader (Perkin-Elmer, Boston, MA).

Table 1. Clinical data for patient samples with AML

Patient no.	Diagnosis	Source	PB-Blast%	BM-blast%	WBC	FAB	Cytogenetics	Status
1	AML	BM	11	57	1.1	OD	Diploid	Primary refractory
2	AML	BM	39	61	3.1	M1	Diploid	Relapsed
3	APL	BM	0% blasts, 82% promyelocytes	7% blasts, 80% promyelocytes	58.3	M3	Pseudodiploid clone 46,XY,t(15;17) (q22;q21) [8] Diploid [11]	New
4	AML	PB	66		1.9	MI	Hypodiploid clone 45,XY,der(12)t(12;17) (p13;q11.2),-7[20]	Primary refractory
5	AML	BM	74	85	94.5	M5B	Pseudodiploid clone 46,XY,t(6;11) (q27;q23) [20]	Relapsed
6	AML	BM	73	95	87.8	OD	Insufficient yield of analyzable metaphases Ph+clone 46,XY,t(2;7) (p21;p22),t(9;22) (q34;q11.2), t(15;17) (q22;q23), del(20) (q11.2) [5]	Relapsed refractory
7	AML	BM	88	93	38.9	M4	Diploid	New
8	AML	BM	78	93	52.5	M4	Pseudodiploid clone 46,XX,del(9) (q22q33) [19]	Relapsed
9	AML	BM	71.8	62	20	M1	Hypodiploid metaphase 45,XX,-7[1],Two pseudodiploid clones 46,XX,-7,+mar[5], 46,XX,t(4;15) (p14;q24), -7,+mar[6],45-46,XX, -7,t(4;15) (p14;q24), -7,-15,+0-5mar[cp8]	Relapsed refractory
10	AML	BM	84	84	16	M1	Diploid	New
11	AML	BM	78	94	6.2	M1	Two hyperdiploid clones 47,XY,+mar[8],48,XY, +8,add(21) (p11.2), +add(21) (p11.2) [9], Diploid[3]	Relapsed refractory
12	AML	BM	94	90	82	M5a	Diploid	New
13	AML	BM	79	83	121.7	OD	Diploid	Relapsed (2nd relapse)

Abbreviations: Ph, pheresis; PB, peripheral blood; BM, bone marrow; WBC, white blood cells count; FAB, French-American-British; OD, outside diagnosis.

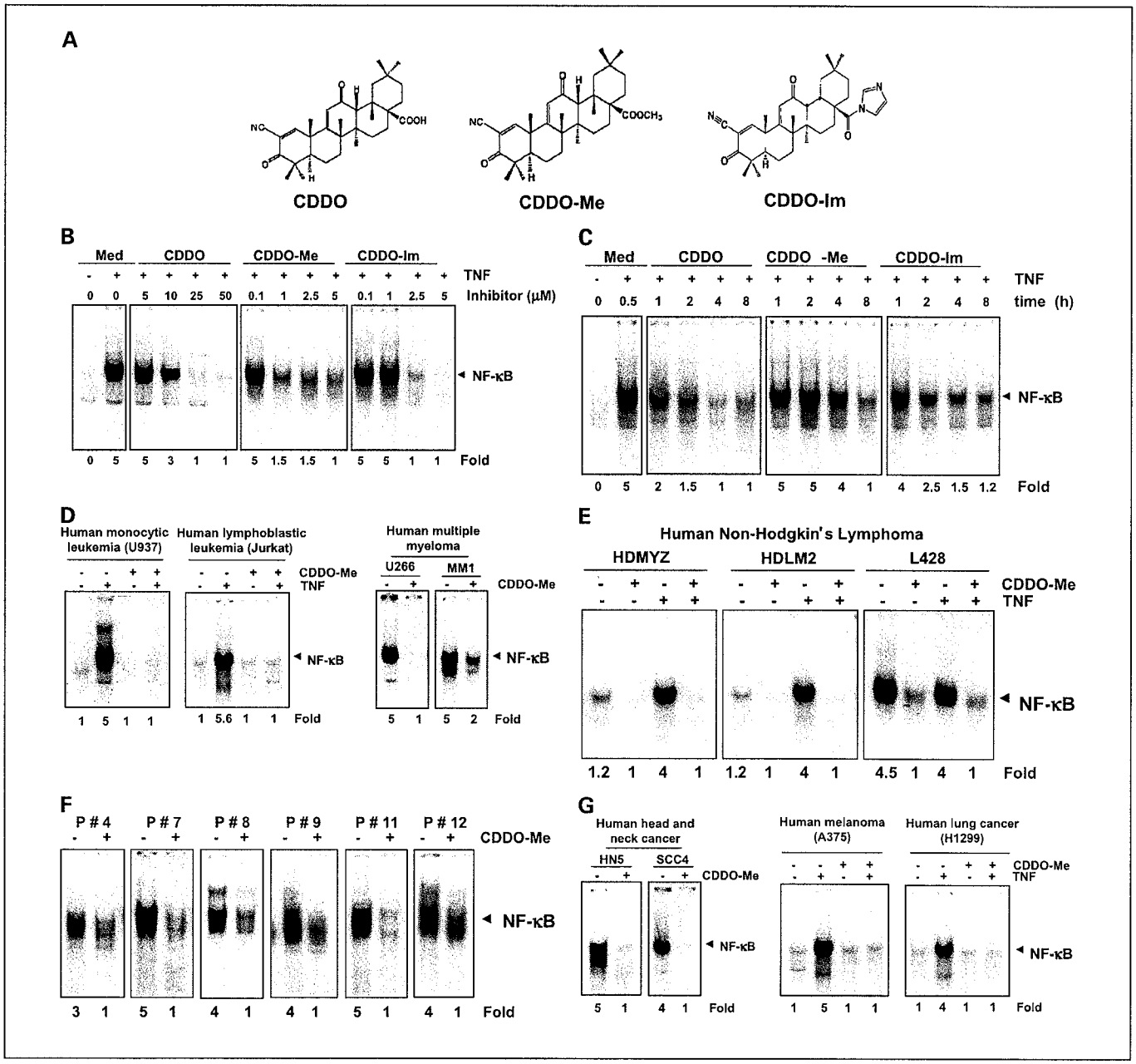

Fig. 1. *A*, structure of CDDO, CDDO-Me, and CDDO-Im. *B*, CDDO, CDDO-Me, and CDDO-Im inhibit TNF-dependent NF-κB activation in a dose-dependent manner. KBM-5 cells (2×10^6/mL) were preincubated with the indicated concentrations of CDDO, CDDO-Me, or CDDO-Im for 8 hours at 37°C and then treated with 0.1 nmol/L TNF for 30 minutes. Nuclear extracts were prepared and tested for NF-κB activation as described in Materials and Methods. *C*, CDDO, CDDO-Me, and CDDO-Im inhibit TNF-dependent NF-κB activation in a time-dependent manner. KBM-5 cells (2×10^6/mL) were preincubated with CDDO (25 μmol/L), CDDO-Me (1 μmol/L), and CDDO-Im (2.5 μmol/L) for the indicated times at 37°C and then treated with 0.1 nmol/L TNF for 30 minutes at 37°C. Nuclear extracts were prepared and then tested for NF-κB activation. *D*, CDDO-Me suppresses NF-κB activation in a non cell-type specific manner. Two million U937, Jurkat, U266, or MM1 cells were pretreated with 1 μmol/L CDDO-Me for 8 hours and then U937 and Jurkat cells were treated with 0.1 nmol/L TNF for 30 minutes as indicated. The nuclear extracts were then prepared and assayed for NF-κB by electrophoretic mobility shift assay as described in Materials and Methods. *E*, two million human non-Hodgkin's lymphoma cells were exposed to 1 μmol/L CDDO-Me for 8 hours, and then nuclear extracts were prepared and assayed for NF-κB by electrophoretic mobility shift assay as described in Materials and Methods. *F*, two million blast cells from AML patients were exposed to 1 μmol/L CDDO-Me for 8 hours, and then nuclear extracts were prepared and assayed for NF-κB by electrophoretic mobility shift assay as described in Materials and Methods. *G*, two million HN5, SCC4, A375, or H1299 cells were pretreated with 1 μmol/L CDDO-Me for 8 hours and then A375 and H1299 cells were treated with 0.1 nmol/L TNF for 30 minutes as indicated, and then nuclear extracts were prepared and assayed for NF-κB by electrophoretic mobility shift assay as described in Materials and Methods.

COX-2 promoter–dependent reporter luciferase gene expression. To determine the effect of CDDO-Me on *cox-2* promoter activity, a *cox-2* reporter assay was done as previously described (27). Briefly, A293 cells were seeded at a concentration of 1.5×10^5 per well in six-well plates. After overnight culture, the cells in each well were transfected with 2 μg of DNA consisting of *COX-2* promoter-luciferase reporter plasmid, along with FuGENE6 reagent, according to the protocol of the manufacturer. After a 24-hour exposure to the transfection mixture, the cells were incubated in medium containing CDDO-Me for 12 hours. The cells were exposed to TNF (0.1 nmol/L) for 24 hours and then harvested. Luciferase activity was measured by using the Luclite (Perkin-Elmer) luciferase assay system according to the protocol of the

manufacturer and detected with a luminometer (Victor 3, Perkin-Elmer). All experiments were done in triplicate and repeated at least twice to prove their reproducibility.

Cytotoxicity assay. Cytotoxicity was assayed by the modified tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as previously described (28). Briefly, 5,000 cells were treated in triplicate in 96-well plates at 37°C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution was then added to each well. After a 2-hour incubation at 37°C, extraction buffer (20% SDS, 50% dimethylformamide) was added, the cells were incubated overnight at 37°C, and the absorbance was then measured at 570 nm using a 96-well multiscanner (Dynex Technologies, MRX Revelation, Chantilly, VA).

Polyadenosine ribose polymerase cleavage assay. For detection of cleavage products of polyadenosine ribose polymerase, whole-cell extracts were prepared by subjecting CDDO-Me-treated cells to lysis in lysis buffer [20 mmol/L Tris (pH 7.4), 250 mmol/L NaCl, 2 mmol/L EDTA (pH 8.0), 0.1% Triton X-100, 0.01 μg/mL aprotinin, 0.005 μg/mL leupeptin, 0.4 mmol/L phenylmethylsulfonyl fluoride, and 4 mmol/L $NaVO_4$]. Lysates were spun at 14,000 rpm for 10 minutes to remove insoluble material, resolved by 10% SDS-PAGE, and probed with polyadenosine ribose polymerase antibodies.

Live and dead assay. To measure apoptosis, we used the Live and Dead assay (Molecular Probes, Carlsbad, CA) as previously described (28). Briefly, 1×10^5 cells were incubated with 1 μmol/L CDDO-Me, alone or in combination with 1 nmol/L TNF, for 16 hours at 37°C. Cells were stained with the Live and Dead reagent (5 μmol/L ethidium homodimer, 5 μmol/L calcein-AM) and then incubated at 37°C for 30 minutes. Cells were analyzed under a fluorescence microscope (Labophot-2, Nikon).

Results

We investigated the effect of a synthetic triterpenoid, CDDO, and its methyl ester and imidazole analogues on the NF-κB-regulated gene products and the NF-κB signaling pathway in a variety of leukemia cell lines and primary blast cells from patients with acute myelogenous leukemia (AML; see Table 1), as well as in other tumor cell lines. Although all the derivatives of CDDO tested (Fig. 1A) were effective in suppressing NF-κB activation, CDDO-Me was the most effective among the three; thus, we examined the effect of this particular compound on NF-κB signaling pathway. The concentration of CDDO-Me used and the duration of exposure for DNA-binding and Western blot analysis had minimal effect on the viability of these cells as determined by trypan blue dye exclusion test (data not shown). To determine the mechanism of action of CDDO-Me, human chronic myelogenous leukemia KBM-5 cells were used. We used TNF to activate NF-κB because the NF-κB signaling pathway activated by TNF is well understood.

CDDO, CDDO-Me, and CDDO-Im block NF-κB activation in a dose- and time-dependent manner. We first examined all the three compounds for their effectiveness in blocking TNF-induced NF-κB activation. As shown by electrophoretic gel shift mobility assay, all of them inhibited NF-κB activation in a dose- and time-dependent manner; however, CDDO-Me was the most potent among the three (Fig. 1B and C). At a dose of 1 μmol/L, CDDO-Me inhibited >90% of NF-κB activation, whereas at this concentration, CDDO and its imidazole analogues were minimally active (Fig. 1B). None of these synthetic triterpenoids activated NF-κB by themselves. Although 25 μmol/L CDDO was sufficient to inhibit NF-κB activation within 1 hour, CDDO-Me required only 1 μmol/L, the lowest among the three. Therefore, we used CDDO-Me for the remainder of the study.

Inhibition of inducible NF-κB activation by CDDO-Me is not cell type specific. It has been reported that the mechanism of NF-κB induction varies among different cell types (29); thus, we examined whether CDDO-Me was effective in blocking NF-κB activation in five human cell lines from a variety of tumors: monocytic leukemia (U937), lymphoblastic leukemia (Jurkat), non-Hodgkin's lymphoma, melanoma (A375), and non-small-cell lung carcinoma (H1299). CDDO-Me completely inhibited TNF-induced NF-κB activation in all five cell lines [Fig. 1D (*left*), 1E (*left* and *middle*), and 1G (*right*)]. Thus, CDDO-Me was effective in inhibiting TNF-inducible NF-κB in a wide variety of tumor types.

CDDO-Me inhibits constitutive NF-κB activation. It has been reported that NF-κB is constitutively active in a wide variety of tumor cells (7). We therefore examined the effect of CDDO-Me in a number of tumor cells where NF-κB is known to be constitutively active. CDDO-Me inhibited the constitutive NF-κB activation in human multiple myeloma (U266 and MM1, Fig. 1D, *right*), non-Hodgkin's lymphoma (L428, Fig. 1E), and human head and neck squamous cell carcinoma cell lines (Fig. 1G, *left*). CDDO-Me also inhibited constitutively active NF-κB in blast cells from AML patients (Fig. 1F). Thus, the NF-κB inhibitory activity of CDDO-Me was not cell type specific.

CDDO-Me blocks NF-κB activation induced by various agents. A wide variety of cytokines, tumor promoters, oxidative stress inducers, and carcinogens have been shown to induce the activation of NF-κB through mechanisms that differ. We therefore examined the effect of CDDO-Me on the activation of NF-κB induced by cigarette smoke condensate, phorbol myristate acetate, okadaic acid, H_2O_2, lipopolysaccharide, IL-1β, and TNF in KBM-5 cells. DNA-binding assay (electrophoretic mobility shift assay) showed that CDDO-Me suppressed the NF-κB activation induced by all these agents (Fig. 2A). These results suggest that CDDO-Me acted at a step in the NF-κB activation pathway that is common to all these agents.

Suppressed NF-κB consists of both p50 and p65. The NF-κB family of proteins comprises five members that combine in different sets to exert a wide variety of effects. The most predominant combination involved in tumorigenesis is a heterodimer of p50(NF-κB1) and p65(RelA). When nuclear extracts from TNF-activated cells were incubated with antibodies to p50 and p65 subunits of NF-κB, the resulting bands were shifted to higher molecular masses (Fig. 2B), suggesting that the TNF-activated complex consisted of p50 and p65. Preimmune serum did not have any effect. Addition of excess unlabeled NF-κB (cold oligo; 100-fold) caused complete disappearance of the band whereas mutated oligo had no effect on the DNA-binding. These results suggest that the NF-κB combination suppressed by CDDO-Me was composed of the p50 and p65 subunits.

CDDO-Me does not directly affect binding of NF-κB to the DNA. Because some NF-κB inhibitors, including *N*-tosyl-L-phenylalanine chloromethyl ketone (the serine protease inhibitor), herbimycin A (protein tyrosine kinase inhibitor), and caffeic acid phenethyl ester, directly modify NF-κB to suppress its DNA binding (30-32), we investigated whether CDDO-Me

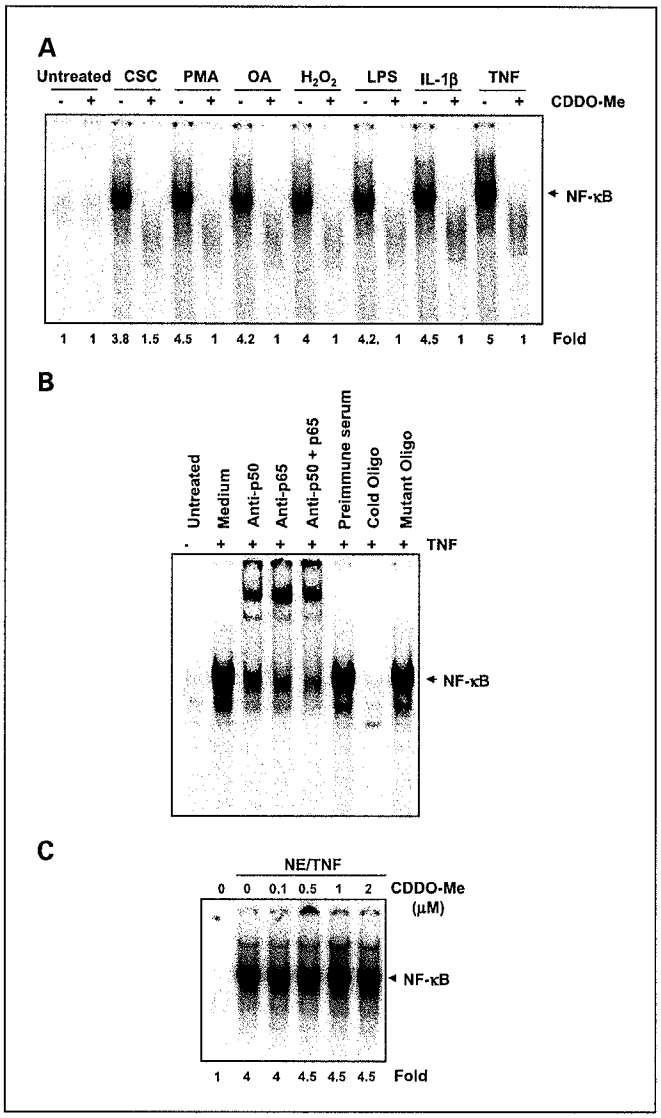

Fig. 2. *A*, CDDO-Me blocks NF-κB activation induced by cigarette smoke condensate (*CSC*), phorbol myristate acetate (*PMA*), okadaic acid (*OA*), H_2O_2, lipopolysaccharide (*LPS*), IL-1β, and TNF. KBM-5 cells (2×10^6/mL) were preincubated for 8 hours at 37°C with 1 μmol/L CDDO-Me and then treated with cigarette smoke condensate (10 μg/mL, 30 minutes), phorbol myristate acetate (100 ng/mL, 1 hour), okadaic acid (500 nmol/L, 4 hours), H_2O_2 (250 μmol/L, 1 hour), lipopolysaccharide (10 μg/mL), IL-1β (100 ng/mL), or TNF (0.1 nmol/L). Nuclear extracts were prepared and tested for NF-κB activation as described in Materials and Methods. *B*, TNF-induced NF-κB consists of p50 and p65 subunits. Nuclear extracts from KBM-5 cells (2×10^6/mL), treated or not treated with 0.1 nmol/L TNF for 30 minutes, were incubated with the antibodies indicated for 30 minutes at room temperature, and the complex was analyzed by supershift assay. *C*, CDDO-Me does not modulate the ability of NF-κB to bind to the DNA. Nuclear extracts from KBM-5 cells (2×10^6/mL), treated or not treated with 0.1 nmol/L TNF for 30 minutes, were treated with the indicated concentrations of CDDO-Me for 2 hours at room temperature and then assayed for DNA binding by electrophoretic mobility shift assay.

also followed a similar mechanism. We did an *in vitro* NF-κB-DNA-binding assay in the presence of various concentrations of CDDO-Me. The dose of CDDO-Me used in our experiments did not directly modify the DNA-binding ability of NF-κB proteins prepared from TNF-treated cells; thus, CDDO-Me seemed to inhibit NF-κB activation by a mechanism other than direct binding (Fig. 2C).

CDDO-Me inhibits TNF-induced IKK activation. Activation of IKK has been shown to be critical for TNF-induced NF-κB activation. As shown in Fig. 3A (*top*), CDDO-Me completely suppressed TNF-induced activation of IKK. Neither TNF nor CDDO-Me had any direct effect on the expression of IKK proteins (Fig. 3A, *middle* and *bottom*).

Next, we examined whether CDDO-Me directly interacted with IKK to block its activity *in vitro*. An *in vitro* kinase assay of TNF-treated extracts in the presence of various concentrations of CDDO-Me showed that CDDO-Me did not directly interfere with the IKK activity (Fig. 3B). Because cellular treatment inhibits IKK activity, CDDO-Me could possibly block the upstream kinases that have been implicated in the activation of IKK.

CDDO-Me inhibits TNF-dependent IκBα phosphorylation. Because IκBα phosphorylation is required for NF-κB activation, we next determined whether CDDO-Me affected TNF-induced IκBα phosphorylation, another condition for NF-κB translocation. Western blot analysis using antibody that detects only the serine-phosphorylated form of IκBα indicated that CDDO-Me completely suppressed TNF-induced IκBα phosphorylation (Fig. 3C). Thus, CDDO-Me inhibited TNF-induced NF-κB activation by inhibiting phosphorylation of IκBα.

CDDO-Me inhibits TNF-dependent IκBα degradation. Because IκBα degradation is typically required for translocation of NF-κB to the nucleus (33), we determined whether inhibition of TNF-induced NF-κB activation by CDDO-Me was due to inhibition of IκBα degradation. We found that TNF induced IκBα degradation in control cells and CDDO-Me completely blocked TNF-induced IκBα degradation (Fig. 3D).

CDDO-Me inhibits TNF-induced phosphorylation of p65. Phosphorylation of p65 is also required for the transcriptional activity of NF-κB (34). Therefore, we also tested the effect of CDDO-Me on TNF-induced phosphorylation of p65. CDDO-Me almost completely suppressed the phosphorylation of p65 (Fig. 3E, *top*).

CDDO-Me inhibits TNF-induced nuclear translocation of p65. Western blot analysis of nuclear extracts (Fig. 3E, *middle*) and an immunocytochemical assay (Fig. 3F) indicated that CDDO-Me inhibited TNF-induced nuclear translocation of p65. We conclude that CDDO-Me inhibited the phosphorylation as well as the nuclear translocation of the p65 subunit of NF-κB.

CDDO-Me represses TNF-induced NF-κB-dependent reporter gene expression. Our results up to this point show that CDDO-Me inhibited the translocation of p65 into the nucleus. Because DNA binding alone does not always correlate with NF-κB-dependent gene transcription, suggesting that there are additional regulatory steps (35), we assayed NF-κB-dependent gene transcription using the SEAP reporter construct. We transiently transfected A293 cells with the NF-κB-regulated construct and then stimulated them with TNF. TNF produced an almost 18-fold increase in SEAP activity over vector control (Fig. 4A), which was abolished by dominant-negative IκBα, indicating specificity. When the cells were pretreated with CDDO-Me, TNF-induced NF-κB-dependent SEAP expression was inhibited in a dose-dependent manner. These results show that CDDO-Me inhibits NF-κB-dependent reporter gene expression induced by TNF.

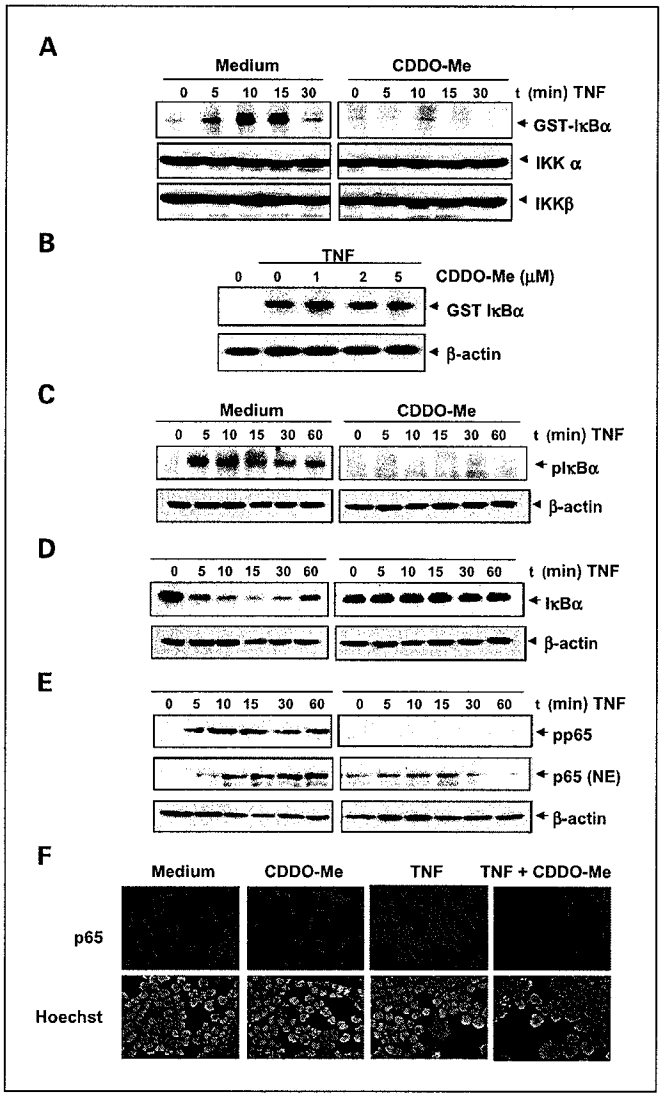

Fig. 3. *A*, CDDO-Me inhibits TNF-induced IκBα kinase activity. KBM-5 cells ($2\times10^6/10^6/10^6$/mL) were treated with 1 μmol/L CDDO-Me for 8 hours and then treated with 0.1 nmol/L TNF for the indicated time intervals. Whole-cell extracts were prepared and 200 μg of extract were immunoprecipitated with antibodies against IKKα and IKKβ. Thereafter, immune complex kinase assay was done as described in Materials and Methods. To examine the effect of CDDO-Me on the level of expression of IKK proteins, 30 μg of whole-cell extract were run on 10% SDS-PAGE, electrotransferred, and immunoblotted with indicated antibodies as described in Materials and Methods. *B*, CDDO-Me has no direct effect on IKK activity. Whole-cell extracts were prepared from untreated and TNF (0.1 nmol/L) – treated KBM-5 cells (2×10^6/mL); 200 μg of protein/sample whole-cell extract were immunoprecipitated with antibodies against IKKα and IKKβ. The immune complex was treated with the indicated concentrations of CDDO-Me for 30 minutes at 30°C, and then a kinase assay was done as described in Materials and Methods. Equal protein loading was evaluated by IKKβ. *C*, CDDO-Me inhibits TNF-induced phosphorylation of IκBα. KBM-5 cells (2×10^6/mL) were incubated with 1 μmol/L CDDO-Me for 8 hours at 37°C, treated with 0.1 nmol/L TNF for the indicated times at 37°C, and then tested for phosphorylated IκBα in cytosolic fractions by Western blot analysis. Equal protein loading was evaluated by β-actin (*bottom*). *D*, CDDO-Me inhibits TNF-induced degradation of IκBα. KBM-5 cells (2×10^6/mL) were incubated with 1 μmol/L CDDO-Me for 8 hours at 37°C, treated with 0.1 nmol/L TNF for the indicated times at 37°C, and then tested for IκBα degradation in cytosolic fractions by Western blot analysis. Equal protein loading was evaluated by β-actin (*bottom*). *E*, CDDO-Me inhibits TNF-induced phosphorylation and translocation of p65. KBM-5 cells (2×10^6/mL) were incubated with 1 μmol/L CDDO-Me for 8 hours and then treated with 0.1 nmol/L TNF for the indicated times. The cytoplasmic extracts were analyzed by Western blotting using antibodies against the phosphorylated form of p65 (*top*) and nuclear extracts were analyzed using antibodies against p65 (*middle*). β-Actin was examined to show equal protein loading. *F*, CDDO-Me inhibits TNF-induced nuclear translocation of p65. KBM-5 cells (1×10^6/mL) were first treated with 1 μmol/L CDDO-Me for 8 hours at 37°C and then exposed to 0.1 nmol/L TNF for 30 minutes. After cytospin, immunocytochemical analysis was done as described in Materials and Methods.

CDDO-Me represses NF-κB-dependent reporter gene expression induced by TNF receptor 1, TNF receptor-asociated death domain, TRAF2, NF-κB-inducing kinase, and IKK. We next determined where CDDO-Me acts in the sequence of TNF receptor 1, TNF receptor-asociated death domain (TRADD), TRAF2, NF-κB-inducing kinase (NIK), and IKK recruitment that characterizes TNF-induced NF-κB activation (36, 37). In cells transfected with TNF receptor 1, TRADD, TRAF2, NIK, IKKβ, and p65 plasmids, NF-κB-dependent reporter gene expression was induced; CDDO-Me suppressed SEAP expression in all cells except those transfected with p65 (Fig. 4B). Because IKK activation can cause the phosphorylation of IκBα and p65, we suggest that CDDO-Me inhibits NF-κB activation through inhibition of IKK.

CDDO-Me represses TNF-induced COX-2 promoter activity. COX-2 promoter has been shown to be regulated by NF-κB (38); thus, we next determined the effect of CDDO-Me on NF-κB-regulated COX-2 promoter activity. As shown in Fig. 4C, CDDO-Me abolished the TNF-induced COX-2 promoter activity in a dose-dependent manner.

CDDO-Me inhibits TNF-induced COX-2, MMP-9, and vascular endothelial growth factor expression. COX-2, MMP-9, and vascular endothelial growth factor (VEGF) are known to be regulated by NF-κB (38-40); thus, the effect of CDDO-Me on the expression of these NF-κB-regulated genes was also examined. TNF treatment induced the expression of COX-2, MMP-9, and VEGF gene products and CDDO-Me abolished the TNF-induced expression of these gene products (Fig. 5A).

CDDO-Me inhibits TNF-induced cyclin D1 and c-myc expression. Both c-myc and cyclin D1 regulate cellular proliferation and are regulated by NF-κB (41). Whether CDDO-Me controls the expression of these gene products was also examined. Our results show that CDDO-Me abolished in a dose-dependent fashion the TNF-induced expression of cyclin D1 and c-myc (Fig. 5B).

CDDO-Me inhibits TNF-induced activation of antiapoptotic gene products. NF-κB up-regulates the expression of a number of genes implicated in facilitating tumor cell survival, including *cIAP2*, *cFLIP*, *TRAF1*, *survivin*, and *bcl-2* (42-50). We found that CDDO-Me inhibited the TNF-induced expression of all of these antiapoptotic proteins (Fig. 5C).

We next examined whether CDDO-Me inhibited the expression of these antiapoptotic proteins in blast cells derived from AML patients. We found that the expression of cIAP1, cFLIP, and Bcl-2 was down-regulated by CDDO-Me (Fig. 5D).

CDDO-Me potentiates the cytotoxic effects of TNF. Because NF-κB-regulated gene products suppress TNF-induced apoptosis (51), we examined the effects of CDDO-Me on the apoptotic effects of TNF. TNF by itself did not induce a significant amount of apoptosis; however, in combination with CDDO-Me, the cytotoxic effects of TNF were enhanced (Fig. 6A, *top left*). CDDO-Me also potentiated the caspase-induced cleavage of polyadenosine ribose polymerase activated by TNF (Fig. 6B).

We next determined the effect of CDDO-Me on TNF-induced cytotoxicity using the Live and Dead assay that determines intracellular esterase activity and plasma membrane integrity. Our result showed that CDDO-Me enhanced the cytotoxic effects of TNF (Fig. 6C).

CDDO-Me potentiates the cytotoxic effects of chemotherapeutic drugs. Chemotherapy-induced apoptosis has also been shown to be suppressed by NF-κB-regulated gene products

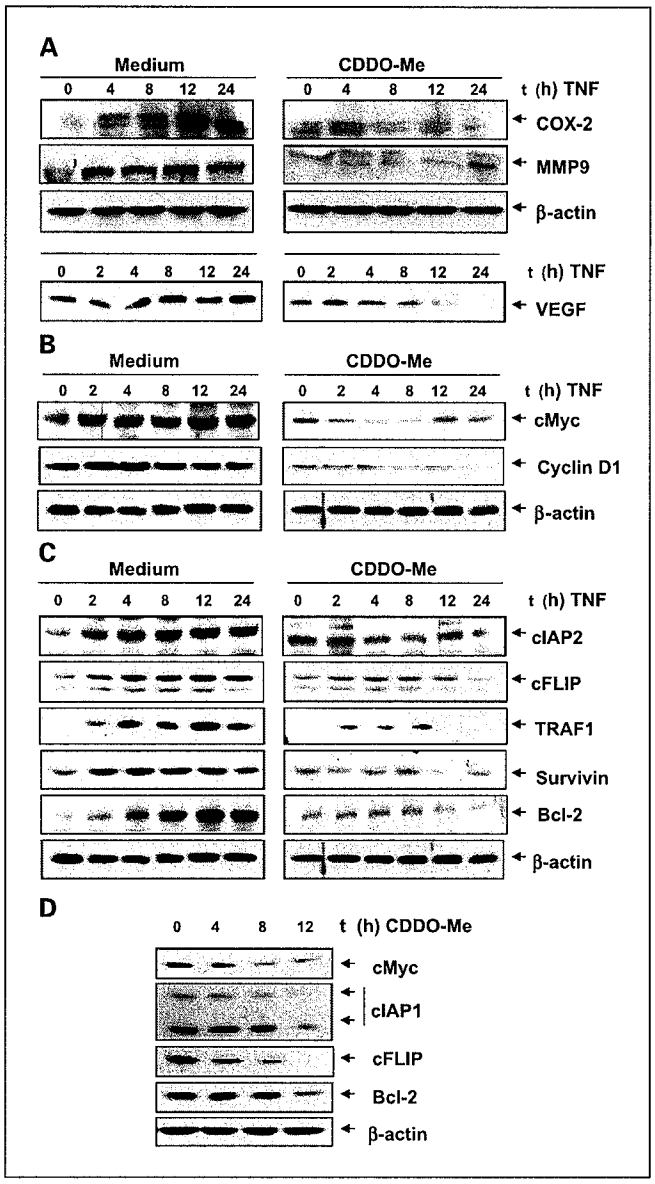

Fig. 5. *A,* CDDO-Me inhibits MMP-9, COX-2, and VEGF expression induced by TNF. KBM-5 cells (2×10^6/mL) were left untreated or incubated with 1 μmol/L CDDO-Me for 8 hours and then treated with 0.1 nmol/L TNF for different times. Whole-cell extracts were prepared and 50 μg of the whole-cell lysate were analyzed by Western blotting using antibodies against VEGF, MMP-9, and COX-2. *B,* CDDO-Me inhibits cyclin D1 and c-myc expression induced by TNF. KBM-5 cells (2×10^6/mL) were left untreated or incubated with 1 μmol/L CDDO-Me for 8 hours and then treated with 0.1 nmol/L TNF for different times. Whole-cell extracts were prepared and 50 μg of the whole-cell lysate were analyzed by Western blotting using antibodies against cyclin D1 and c-myc. *C,* CDDO-Me inhibits the expression of antiapoptotic gene products cIAP2, cFLIP, TRAF1, survivin, and Bcl-2. KBM-5 cells (2×10^6/mL) were left untreated or incubated with 1 μmol/L CDDO-Me for 8 hours and then treated with 0.1 nmol/L TNF for different times. Whole-cell extracts were prepared and 50 μg of the whole-cell lysate were analyzed by Western blotting using antibodies against proteins as indicated. *D,* CDDO-Me inhibits the expression of antiapoptotic gene products cIAP1, cFLIP, and Bcl-2 in blast cells from AML patients. AML blast cells (2×10^6/mL) were incubated with 1 μmol/L CDDO-Me for different time points as indicated. Whole-cell extracts were prepared and 50 μg of the whole-cell lysate were analyzed by Western blotting using antibodies against proteins as indicated.

Besides suppressing activation of constitutively expressed NF-κB, CDDO-Me also suppressed NF-κB activation induced by carcinogens, tumor promoters, and inflammatory stimuli in leukemia cells. Because the pathway through which these various agents activate NF-κB may vary, these results suggest that CDDO-Me must act at a common step. We found that CDDO-Me suppressed the TNF-induced phosphorylation and degradation of IκBα, leading to inhibition of p65 translocation into the nucleus. Our results differ from that of Stadheim et al. (59) who showed that CDDO did not affect the TNF-induced phosphorylation and degradation of IκBα or nuclear p65 translocation. Their results, however, suggest that CDDO inhibits NF-κB-dependent IκBα resynthesis at a level downstream of p65(RelA) accumulation in the nucleus by exerting a destabilizing effect on specific mRNA transcripts. In their study, authors did not investigate the effect of CDDO on TNF-induced NF-κB DNA binding or on the NF-κB reporter assays. Whether

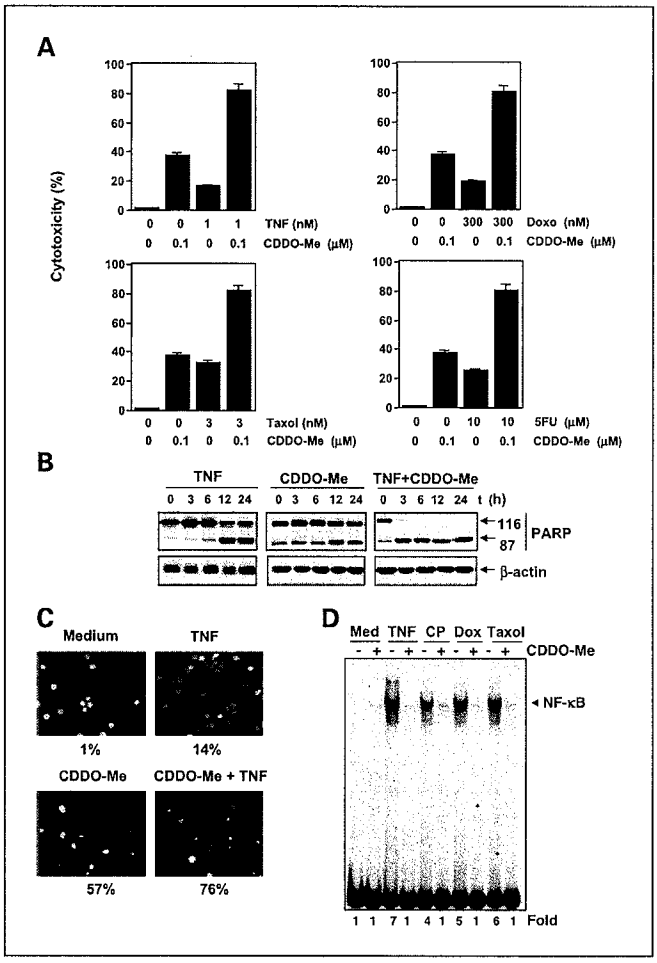

Fig. 6. CDDO-Me enhances apoptosis induced by TNF and chemotherapeutic agents. *A,* KBM-5 cells (5,000/0.1 mL) were incubated at 37°C with TNF, Taxol, 5-fluorouracil (*5-FU*), or doxorubicin in the presence and absence of 0.1 μmol/L CDDO-Me, as indicated, for 72-hour duration, and the viable cells were assayed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent. Columns, mean cytotoxicity from triplicate cultures; bars, SD. *B,* KBM-5 cells (2×10^6/mL) were serum starved for 24 hours and then incubated with TNF (1 nmol/L), alone or in combination with CDDO-Me (1 μmol/L), for the indicated times and polyadenosine ribose polymerase (*PARP*) cleavage was determined by Western blot analysis as described in Materials and Methods. *C,* KBM-5 cells (2×10^6/mL) were serum starved for 24 hours and then incubated with TNF (1 nmol/L), alone or in combination with CDDO-Me (0.1 μmol/L), as indicated for 24 hours. Cell death was determined by calcein-AM – based Live and Dead assay as described in Materials and Methods. *D,* KBM-5 cells (2×10^6/mL) were preincubated with CDDO-Me (1 μmol/L) for 8 hours and then treated with chemotherapeutic agents, cisplatin (*CP*; 20 μg/mL, 4 hours), doxorubicin (*Dox*; 50 μmol/L, 4 hours), and Taxol (50 μmol/L, 16 hours) at 37°C. Nuclear extracts were prepared and then tested for NF-κB activation.

the differences in results are due to cell type, dose, or time of exposure to CDDO is unclear.

We found that CDDO-Me also inhibited TNF-induced activation of IKK. Our *in vitro* kinase assay results show that CDDO-Me is not a direct inhibitor of IKK. Thus, it seems that CDDO-Me blocks the activation of IKK indirectly by interfering with some upstream regulatory kinases; this possibility requires further investigation. Akt, NIK, mitogen-activated protein kinase kinase kinase 1, and atypical protein kinase C are the potential candidates because they are some of the upstream kinases that regulate IKK (60–62).

Besides NF-κB activation, the NF-κB reporter activity induced by TNF, TNF receptor 1, TRADD, TRAF2, NIK, and IKK was also abrogated by CDDO-Me. The NF-κB reporter activity induced by p65, however, was unaffected by CDDO-Me, suggesting that this agent acts upstream to p65. NF-κB activation leads to the expression of genes that are involved in the proliferation and metastasis of cancer (13, 14, 40). In this report, we show that CDDO-Me inhibits the expression of cyclin D1 and c-myc, which are both regulated by NF-κB. Our results are also consistent with a recent report by Lapillonne et al. (63) who showed that CDDO down-regulates cyclin D1 and Bcl-2.

Our results also show that the expression of COX-2, MMP-9, and VEGF, also known to be regulated by NF-κB, is down-regulated by CDDO-Me. Our observations are consistent with previous reports that CDDO suppresses the abilities of various inflammatory cytokines, such as IFNγ, IL-1, and TNF, to induce *de novo* formation of the enzymes inducible nitric oxide synthase and inducible COX-2 in mouse peritoneal macrophages, rat brain microglia, and human colon fibroblasts (20). CDDO inhibits IL-1-induced MMP-1 and MMP-13 expression in human chondrocytes. CDDO also inhibits the expression of Bcl-3, an IL-1-responsive gene that preferentially contributes to MMP-1 gene expression (64). These results further imply that CDDO-Me exercises its anticancer properties through the inhibition of NF-κB.

NF-κB is known to regulate the expression of IAP1, X-linked IAP, Bfl-1/A1, TRAF1, Bcl-2, cFLIP, and survivin, and their overexpression in numerous tumors has been linked to tumor cell survival, chemoresistance, and radioresistance. Our results indicate that CDDO-Me treatment down-regulates all of these gene products. CDDO-Me has been shown to inhibit the growth of wide variety of tumor cells including leukemic cells and non–small-cell lung carcinoma cells (21, 54, 65). This inhibition of growth may be mediated through down-regulation of various genes as shown here. Down-regulation of cFLIP by CDDO-Me as described here is consistent with a previous report in AML described using CDDO (22).

We found that CDDO-Me also suppressed the NF-κB activation induced by the chemotherapeutic drugs, cisplatin, doxorubicin, and Taxol, and potentiated apoptosis. The down-regulation of various antiapoptotic gene products by CDDO-Me also sensitized the cells to the apoptotic effects of TNF. The sensitization of leukemic and lung cancer cell lines to TRAIL by CDDO (22, 66) may also be due to down-regulation of various gene products as reported here. Overall, our results indicate that apoptotic, antiproliferative, anti-invasive, anti-angiogenic, antimetastatic, and anti-inflammatory activities assigned to CDDO-Me may be mediated through suppression of NF-κB and NF-κB-regulated gene products.

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Abstract Number:	4955
Presentation Title:	The novel triterpenoid CDDOme potently synergizes with inhibition of bcl-2 function to induce apoptosis in AML via disruption of intracellular redox homeostasis
Presentation Start/End Time:	Tuesday, Apr 19, 2005, 1:00 PM - 5:00 PM
Board Number:	Board #27
Author Block:	*Ismael Samudio*, *Rooha Contractor*, *Marina Konopleva*, *Michael Andreeff*. UT M. D. Anderson Cancer Center, Houston, TX

Small molecule BH3 inhibitors induce apoptosis in AML cell lines and primary samples via activation of the mitochondrial pathway of cell death and have validated bcl-2 as a chemotherapeutic target. CDDOme is a novel triterpenoid that has been shown to induce apoptosis in a variety of cancers via perturbations in intracellular redox tone. Here we report for the first time that CDDOme synergizes with BH3 inhibitors to induce loss of mitochondrial membrane potential ($\Delta\Psi M$), caspase activation, and apoptosis in AML cells abrogating the protective effects of bcl-2 overexpression. Our results demonstrate that depletion of glutathione (GSH) or NADPH exacerbates the cytotoxicity of BH3 inhibitors and potentiates their synergism with CDDOme. Accordingly, apoptosis induced by the combination of CDDOme with BH3 inhibitors is markedly abrogated by cotreatment with the antioxidant n-acetyl cysteine (NAC). We have previously demonstrated that inhibition of MEK activity synergizes with BH3 inhibition to induce apoptosis in AML. Interestingly, treatment of leukemic cells with CDDOme leads to a dose-dependent, redox-dependent inhibition of p42/44 MAPK phosphorylation suggesting that MEK inhibition contributes to the synergism in between CDDOme and BH3 inhibitors. In addition, the rapid decrease in GSH levels induced by CDDOme is potentiated by BH3 inhibitors suggesting that BH3 inhibition modulates the redox capacity of leukemic cells. Taken together our results demonstrate the efficacy of combining BH3 inhibitors and CDDOme, suggest that bcl-2 has redox modulating functions in AML, and demonstrate that agents targeting intracellular redox tone potentiate the effects of BH3 inhibitors.

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